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Influencia del proceso de germinación en el contenido y biodisponibilidad de melatonina en semillas de legumbres

MEMORIA PRESENTADA POR:

Teresa Herrera Rodríguez

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DIRECTORAS DE TESIS:

Dra. María Ángeles Martín Cabrejas

Dra. Yolanda Aguilera Gutiérrez

Dra. María Dolores Del Castillo Bilbao

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MARÍA ÁNGELES MARTÍN CABREJAS, PROFESORA TITULAR DE UNIVERSIDAD, YOLANDA AGUILERA GUTIÉRREZ PROFESORA CONTRATADO DOCTOR, AMBAS DEL DEPARTAMENTO DE QUÍMICA AGRÍCOLA Y BROMATOLOGÍA DE LA FACULTAD DE CIENCIAS DE LA UNIVERSIDAD AUTÓNOMA DE MADRID, Y MARÍA DOLORES DEL CASTILLO BILBAO, INVESTIGADORA CIENTÍFICO DEL CONSEJO SUPERIOR DE INVESTIGACIONES DEL INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

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El presente trabajo titulado "Influencia del proceso de germinación en el contenido de melatonina y biodisponibilidad en semillas de legumbres" ha sido realizado por Dña. Teresa Herrera Rodríguez bajo nuestra dirección en el Departamento de Química Agrícola y Bromatología constituyendo la Tesis Doctoral de su autora. Dicho trabajo reúne las condiciones necesarias para su presentación y defensa.

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Fdo.: Dra. Yolanda Aguilera Gutiérrez

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Facultad de Ciencias
Departamento de Química Agrícola y Bromatología

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Que el trabajo de investigación titulado: "Influencia del proceso de germinación en el contenido de melatonina y biodisponibilidad en semillas de legumbres" se ha realizado en este Departamento bajo la dirección de las Dras. María Ángeles Martín-Cabrejas, Yolanda Aguilera Gutiérrez, y María Dolores del Castillo Bilbao y que constituye la Memoria que presenta Dª Teresa Herrera Rodríguez para optar al Grado de Doctor.

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Fdo. Juan José Lucena Marotta

A mi padre,
mi madre y hermano
A ti, José

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Abreviaturas y Acrónimos

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6-HMEL:	6-hidroximelatonina
ABTS:	2,2-azinobis-[3 etilbenzotiazolin-6-sulfónico]
Ac:	Anticuerpo
AFMK:	N1-acetil-N2-formil-5-metoxikinuremina
Ag:	Antígeno
AMK:	N1-acteil-5-metoxikinuremina
aMT6s:	6-sulfatoximelatonina
ASNMT:	Acetilserotonin N-metiltransferasa)
C3-OHM:	3-hidroximelatonina cíclica
CAT:	Catalasa
CFL	Compuestos fenólicos libres
CFT	Compuestos fenólicos totales
CFU	Compuestos fenólicos unidos
CG-MS:	Cromatografía de gases acoplada a espectometría de masas
DM:	Diabetes Mellitus
DPPH:	2,2-diphenyl-1-picrylhydrazyl
DS:	Sprague-Dawley
EC	Electroforesis capilar
EFSA	Autoridad Europea de Seguridad Alimentaria
EIA:	Enzimoinmunoensayo
ELISA:	Ensayo de inmunoadsorción ligado a enzimas
F.S.:	Frutos secos
FRAP:	ferric reducing/antioxidant power
GAE:	Equivalentes de ácido gálico
GnRH:	Hormona liberadora de gonadotropina
GPx:	Glutatión peroxidasa
GR:	Glutatión reductasa
GSH:	Glutatión reducido
GSSG:	Glutatión oxidado
h:	Hora
HIOMT:	Enzima Hidroxi-indol metiltransferasa
HPLC:	Cromatografía de líquidos alta resolución
HPLC-ECD:	Cromatografía de líquidos alta resolución acoplado a detector electroquímico
HPLC-FL:	Cromatografía de líquidos alta resolución acoplado a espectometría de fluorescencia
HPLC-MS:	Cromatografía de líquidos alta resolución acoplada a espectometría de masas
IAA:	Ácido indol acético
IAAId:	Ácido 3-indolacetaldehido
IAM:	Indol 3-acetamida
IAN:	Indol 3-acetonitrilo
IBA:	Indol-3 butírico

IDO:	Enzima indolamina-2,3-dioxigenasa
IPA:	Ácido indol 3-pirúvico
LC-MS:	Cromatografía líquida con espectometría de masas
LDL:	Lipoproteína de baja densidad
m.s.:	Materia seca
MEL:	Melatonina
min:	Minutos
NAS:	N-acetilserotonina
ORAC:	Capacidad atrapadora de radicales de oxígeno
PAD:	Detector de fotodiodos alineados
RIA:	Radioinmuno ensayo
RNS:	Especies reactivas de nitrógeno
ROS:	Especies reactivas de oxígeno
s:	Semanas
SHR:	Ratas espontáneamente hipertensas
SNAT:	Serotonin N-acetiltransferasa
SOD:	Superóxido dismutasa
SPE:	Extracción en fase sólida
T5H:	Triptófano 5-hidroxilasa
TDA:	Triptamina desaminasa
TDC:	Triptófano descarboxilasa
TE:	Equivalentes de Trolox
TPH:	Triptófano hidrolasa
WKY:	Wistar Kyoto

Resumen

Resumen

Las legumbres contienen sustancias bioactivas conocidas como fitoquímicos con propiedades antioxidantes. El proceso de germinación puede dar lugar a alimentos con características nutricionales y sensoriales mejoradas; así como incrementar sus niveles en antioxidantes. La presente investigación tiene como objetivo principal evaluar la efectividad de la germinación en la mejora del valor nutricional y la biodisponibilidad de antioxidantes de la dieta, concretamente, de la melatonina en legumbres. Hasta la fecha, no existen datos acerca de los niveles de melatonina en semillas de legumbres y hortalizas.

Los estudios realizados han dado lugar a información novedosa y confirman la presencia de melatonina en semillas de hortalizas y legumbres comerciales y sus correspondientes germinados. Los valores más elevados de melatonina se encontraron en germinados de col roja comercial. El proceso de germinación modifica el perfil de los componentes bioactivos, siendo dependiente del tipo de legumbre y de las condiciones en las que se realiza. Las investigaciones realizadas han permitido establecer que la germinación de semillas de lenteja y judía pinta durante 8 días, bajo condiciones lumínicas de 12 horas luz/12 horas oscuridad y/o 24 horas en oscuridad, produce un aumento significativo de los contenidos de melatonina e incrementa su capacidad antioxidante. Los niveles máximos de melatonina en lenteja y judía pinta se logran bajo condiciones de 24 horas en oscuridad en el sexto día de germinación. Los contenidos más elevados se alcanzan en germinados de judía pinta (9,4 ng/g m.s.). Por otra parte, el tratamiento combinado de riego suplementado con melatonina pura durante la germinación resulta ser efectivo en el incremento de melatonina (99%). El incremento se atribuye, al menos en parte, a su absorción. El estudio de biodisponbilidad en ratas Sprague Dawley de la melatonina dietética presente en extractos preparados a partir de germinados de judía pinta, revela un incremento de los niveles de melatonina plasmática de 25 a 30 pg/mL, tras la ingesta de una dosis única del extracto que contiene 10,6 µg de melatonina. En línea con el resultado de melatonina plasmática, la excreción de 6-sulfatoximelatonina, metabolito de degradación de melatonina se incrementa en un orden de cuatro veces tras la ingesta del extracto enriquecido con melatonina, destacando una elevada correlación ($r = 0,73$, $p < 0,01$). Por tanto, los resultados sugieren que la melatonina dietética ha sido absorbida y metabolizada, y se encuentra biodisponible.

En conclusión, se ha logrado establecer condiciones de germinación que permiten mejorar el valor nutricional de las legumbres e incrementar su contenido en fitoquímicos antioxidantes, concretamente en melatonina, hasta alcanzar concentraciones biodisponibles. Dado que se ha encontrado melatonina en germinados

de legumbres y hortalizas, puede decirse que son una fuente dietética de melatonina. En concreto, los extractos de judía pinta germinada podrían considerarse como fuente alimentaria de melatonina y otros compuestos bioactivos y aportar efectos beneficiosos para la salud. No obstante, se requieren de investigaciones clínicas para elucidar si el consumo de melatonina de judía pinta germinada puede asociarse a una mejora en la defensa antioxidante del organismo y reducir el riesgo de enfermedades crónicas asociadas a estrés oxidativo.

Introducción

I. INTRODUCCIÓN

Los alimentos de origen vegetal son de gran interés debido a que contienen un gran número de compuestos bioactivos con propiedades antioxidantes. Muchas de las patologías crónicas que se consideran epidemias del siglo XXI se asocian a estrés oxidativo. Por tanto su consumo rutinario como parte de la dieta es de gran interés. En consecuencia, en el mercado internacional se ha incrementado la presencia de los denominados alimentos funcionales ricos en antioxidantes. Previo a su comercialización con esta alegación sanitaria en Europa debe demostrarse que los alimentos contienen una cantidad del compuesto bioactivo en una dosis fisiológicamente activa. (EFSA Scientific Committee, 2009). En este sentido, los germinados representan una oportunidad. Se requieren de estudios que demuestren su carácter funcional y el presente estudio representa un avance en este conocimiento.

La germinación de alimentos para consumo humano es originaria de países orientales, donde constituye una parte importante de la dieta de la población. Sin embargo, en las últimas décadas los países de la cuenca Mediterránea han cambiado sustancialmente sus hábitos, quedando relegados algunos platos tradicionales como, por ejemplo, las legumbres. Estas nuevas preferencias y necesidades en el mercado han dado lugar a un nuevo perfil del consumidor actual. Los germinados se consumen en crudo como ensaladas o en gran diversidad de platos cocinados, como sopas o rehogados; supone consumir semillas vivas con numerosos enzimas que facilitan la digestión, además son productos de bajo aporte calórico, pero con gran cantidad de compuestos bioactivos por determinar. Entre los alimentos vegetales susceptibles a la germinación destacan las legumbres y hortalizas. Estos alimentos componen un grupo bastante diverso y forman parte esencial de la dieta, aportando numerosos nutrientes con un bajo contenido calórico.

La germinación resulta un método barato y efectivo para mejorar la calidad nutricional y sensorial de las legumbres (Vidal-Valverde y col., 2002; Martín-Cabrejas y col., 2003; Martín-Cabrejas y col., 2008; Donkor y col., 2012; Vaz Patto y col., 2015; Dueñas y col., 2016). Además, no se consideran nuevos alimentos lo que se facilita su comercialización. Por otro lado, el proceso de germinación puede proporcionar a la industria alimentaria un material interesante para su incorporación como ingrediente en la elaboración de alimentos con funcionalidad mejorada y supone una estrategia para incrementar el consumo de legumbres y hortalizas.

1. Germinación

La germinación es un proceso metabólico de enorme complejidad en el que la semilla pasa de un estado quiescente a una fase metabólica activa responsable de proporcionar la energía y nutrientes necesarios para el desarrollo de la nueva planta (Paucar-Menacho y col., 2010). La semilla es un sistema funcional constituido por células meristemáticas de crecimiento reducido o suspendido, reservas de alimento y una cubierta protectora restrictiva, muy importante en la regulación de la germinación (Guevara Berger y Jiménez, 1998). Cuando las condiciones (suelo, humedad y temperatura) son adecuadas para la semilla, comienza el proceso de germinación. Se produce en tres fases: hidratación (Fase I), germinación (Fase II) y crecimiento (Fase III) (Figura 1).

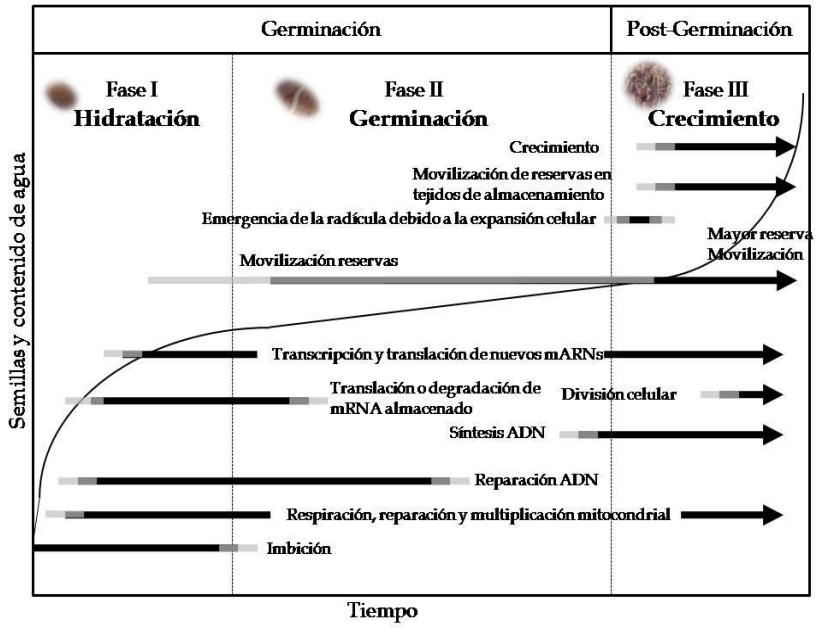


Figura 1. Fases de la germinación.
Fuente: (Nonogaki y col., 2010)

En la fase I, de hidratación, se produce la imbición de agua por la semilla seca, hasta la completa hidratación de células y tejidos, así como un aumento de la actividad respiratoria y reacciones hidrolíticas. Durante la fase II, se produce la activación del metabolismo, dando lugar a las transformaciones necesarias para el desarrollo de la plántula, como la síntesis de ácidos nucleicos y proteínas, también se incrementan las actividades enzimáticas, así como la degradación inicial de las reservas, es un periodo

de absorción de agua limitada. Por último, la fase III, de crecimiento, está asociada a una elevada actividad metabólica debida a la emergencia de la radícula y consecuentemente el resto de tejidos, incrementando la expansión celular, concluyendo el proceso germinativo. Estas fases no definen temporalmente los eventos metabólicos que ocurren durante la germinación, por lo que son procesos confluentes (Nonogaki y col., 2010).

Dentro de los requerimientos ambientales necesarios para la germinación se consideran esenciales el agua, el oxígeno, la luz y la temperatura. La germinación puede modificarse por diversos factores intrínsecos, como son los relacionados con la madurez y la viabilidad de las semillas, y también por factores extrínsecos como humedad, temperatura y concentración de gases (Herrera y col., 2006; Nonogaki y col., 2010). Cada uno estos factores pueden inhibir o estimular la germinación, de forma que una adecuada combinación determinará el desarrollo del proceso.

En este sentido, las condiciones lumínicas son un factor implicado a destacar. La luz es percibida mediante fotorreceptores (fitocromos) por las semillas, de forma que pueden afectar a su regulación y como consecuencia su implicación en procesos metabólicos (Herrera y col., 2006). La cantidad de fitocromo activo presente en una semilla, determina si ésta puede germinar en oscuridad o su necesidad de luz. Se ha observado que las condiciones lumínicas modifican el perfil de compuestos fenólicos, así como el potencial antioxidante, ya que induce la activación de la vía de las pentosas fosfato (Świeca y col., 2012).

2. Cambios en el perfil nutricional y de compuestos bioactivos inducidos por la germinación

Durante la germinación se activan enzimas amilolíticas, lipolíticas y proteolíticas que hidrolizan los compuestos de reserva de la semilla. Se producen modificaciones tales como la composición de carbohidratos solubles, afectando sobre la estructura de los polisacáridos, ya que el almidón se degrada a azúcares más fáciles de digerir. Asimismo, se producen cambios en la integridad de los tejidos y provoca la ruptura de las asociaciones proteína-carbohidratos (Martín-Cabrejas y col., 2003; López-Amorós y col., 2006; Martín-Cabrejas y col., 2008) dando lugar a un aumento de aminoácidos libres, fibra, vitaminas (estimula la biosíntesis de determinadas vitaminas hidrosolubles) y minerales (Fe y Se) (Frías y col., 1996; Martín-Cabrejas y col., 2008; Donkor y col., 2012; Benítez y col., 2013).

La mayor parte de los estudios realizados en germinados de legumbres y hortalizas muestran un aumento de determinados compuestos bioactivos como los compuestos fenólicos totales en presencia o ausencia de luz mejorando así, la funcionalidad de las

semillas (Fernandez-Orozco y col., 2006; Pérez-Balibrea y col., 2008; Shohag y col., 2012; Tajoddin y col., 2014). Se producen modificaciones debido al incremento de compuestos de tipo flavonoideos (Dueñas y col., 2009; Pająk y col., 2014), donde se han observado los cambios más importantes, destacando la formación en judías de glucósidos de flavonoles (López-Amorós y col., 2006) y la disminución de proantocianidinas en lentejas (Bartolomé y col., 1997). En el caso de las isoflavonas de soja, se observó un aumento en el contenido de las agliconas como consecuencia del aumento de la actividad glucosidasa por acción del proceso de germinación (Lin y Lai, 2006). Además se ha observado un aumento generalizado de compuestos hidroxicinámicos y un descenso de los hidroxibenzoicos (López-Amorós y col., 2000). Por otro lado, disminuye el contenido de factores antinutricionales (fitatos, taninos e inhibidores de proteasa) (Osman, 2007; Ghavidel y Prakash, 2007; Steve Ijarotimi y Ruth Esho, 2009), incrementando la digestibilidad de las proteínas, por lo que pueden aumentar la calidad nutricional de los alimentos. Debido a la presencia de estos compuestos, la aplicación de procesos biotecnológicos resulta imprescindible para mejorar su valor nutricional e incrementar el potencial de las leguminosas como alimentos funcionales o ingredientes de alimentos funcionales aportando muchos beneficios en la dieta (Davila y col., 2003). Sin embargo, apenas existen estudios sobre los cambios que sufre la melatonina durante la germinación (Zielinski y col., 2001).

Por lo tanto, la selección de las condiciones óptimas de germinación es muy importante para optimizar el contenido de compuestos bioactivos y actividades biológicas en los germinados.

- ## Semillas de leguminosas

Las legumbres son las semillas secas y limpias procedentes de la familia *Fabaceae*, cuyas características botánicas comunes corresponden a las de la subfamilia "*Papilionadeae*". Esta familia es extraordinariamente rica en especies y enormemente difundida, siendo los suelos preferidos los calcáreos y secos para su desarrollo. Su consumo está distribuido a nivel mundial.

En la alimentación humana actual, más concretamente en la dieta mediterránea, se utilizan hasta 150 especies de legumbres. Las más relevantes son las judías, lentejas, garbanzos, entre otras (Tabla 1). En su composición interesa destacar los elevados contenidos de proteínas, carbohidratos de asimilación lenta (almidón), minerales (Ca, Fe, Zn), fibra dietética (especialmente su contenido en la fracción soluble) y algunos componentes bioactivos minoritarios (Olmedilla Alonso y col., 2010). Por otro lado, las legumbres presentan un bajo contenido en lípidos, constituidos principalmente por ácidos grasos poliinsaturados.

Tabla 1. Composición nutricional (g/100g m.s.) de leguminosas de consumo humano.

	Proteínas	Lípidos	Carbohidratos	Fibra	Minerales	Valor energético (Kcal/100g)
Judía	21,3	1,6	47,8	18,4	4,0	301
Lenteja	23,5	1,4	50,8	10,6	3,2	321
Garbanzo	22,7	3,0	54,6	10,7	3,0	337
Soja	36,9	18,1	6,1	20,9	4,7	357

Fuente: (Hernandez, 2010).

Además existen leguminosas no convencionales utilizadas como recurso alimentario alternativo, dentro de las cuales, se puede destacar las semillas de *Vigna unguiculata*, *Canavalia ensiformis*, *Mucuna pruriens* y *Lablab purpureus*, que son de origen tropical y pertenecen a la familia *Leguminosae* (*Fabaceae*). Al igual que las legumbres convencionales, las no convencionales también poseen un elevado valor nutritivo ya que se caracterizan por contener altas cantidades de proteínas y carbohidratos y por ser una fuente rica en macro y microelementos (Natarajan y col., 2012). Además, suelen ser cultivos de bajo costo, por lo que poseen un gran potencial para la alimentación animal y humana (Chel-Guerrero y col., 2002). Dichas leguminosas tropicales tienen muchas posibilidades agronómicas ya que son muy utilizadas como abonos verdes y cultivos de cobertura mejorando así las condiciones de fertilidad de los suelos agrícolas; de esta manera, se reduce la dependencia de fertilizantes químicos, los cuales son muy costosos. Los principales beneficios obtenidos por su uso son: la fijación de nitrógeno atmosférico, el aporte de biomasa al suelo, el incremento de la actividad biológica y la reducción de la erosión del suelo (Blanchart y col., 2006). Además, estos cultivos se adecuan, en general, a diferentes tipos de suelos y climas, y suelen tolerar bien la sequía.

Entre los compuestos bioactivos presentes en las legumbres podemos encontrar, la melatonina y los compuestos fenólicos. Destaca el nivel de melatonina detectado en la alfalfa (Manchester y col., 2000) aunque apenas ha sido estudiado en el resto de leguminosas. Asimismo, se pueden considerar una fuente importante de compuestos fenólicos, aunque su composición varía notablemente no sólo debido a factores genéticos (especie y variedad), sino también a factores edafoclimáticos durante su cultivo (estado de madurez, condiciones de almacenamiento de la semilla). En las legumbres, se han detectado la mayor parte de las familias de compuestos fenólicos, destacando el contenido de flavonoides, ácidos fenólicos y proantocianidinas (Amarowicz y Pegg, 2008). A modo de ejemplo, la lenteja es rica en flavonoides, destacando su contenido en catequinas y proantocianidinas (Dueñas y col., 2007), mientras la judía es rica en flavonoles (glucósidos de quercetina y glucósidos de kaempferol)(Dinelli y col., 2006) (Figura 2).

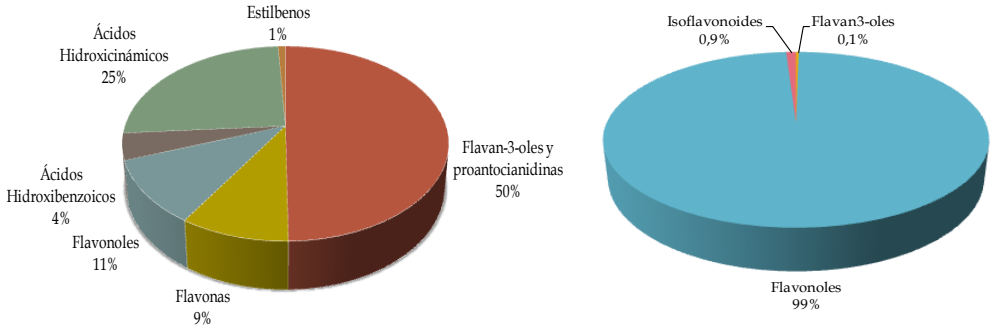


Figura 2. Contenido de compuestos fenólicos en lenteja y judía, respectivamente.
Fuente: (Phenol-Explorer).

Estudios epidemiológicos indican una asociación positiva entre la ingesta diaria de compuestos presentes en legumbres y la prevención de diversas patologías como diabetes, enfermedades cardiovasculares y síndrome metabólico (Rizkalla y col., 2002) . Estos compuestos bioactivos, destacan por su alto contenido antioxidante, lo que está asociado con una menor incidencia de estas enfermedades (Kris-Etherton y col., 2004; Oboh, 2006). Entre los compuestos presentes, destacan los compuestos fenólicos, mencionados anteriormente, con propiedades antiinflamatorias como los flavononoides (Kris-Etherton y col., 2004; Sharma y col., 2011), anticancerígenas y antimutagénicas como las flavonas, flavanonas y lignanos (Gupta y col., 2001; Kanno y col., 2005). Destaca la actividad antioxidantes de estos compuestos y su acción contra radicales libres, y quelantes de iones metálicos (Xu y col., 2007; Djordjevic y col., 2011) protegiendo del estrés oxidativo (Amarowicz y Pegg, 2008; Marathe y col., 2011).

En resumen, las legumbres, han adquirido importancia como ingrediente funcional, debido a que son fuente de proteínas, fibra alimentaria, micronutrientes y compuestos bioactivos contribuyendo a la prevención de enfermedades relacionadas con el estrés oxidativo (Bouchenak y Lamri-Senhadji, 2013).

Mencionar, que la Asamblea General de las Naciones Unidas proclamó este año 2016 el Año Internacional de las Legumbres (Asamblea general (A/RES/68/231) Naciones Unidas,), cuyo objetivo es concienciar de los beneficios de las legumbres, para promover su consumo, aumentar los niveles de nutrición e incrementar la producción.

- **Semillas de hortalizas**

Las hortalizas son aquellas plantas herbáceas hortícolas de las que se pueden consumir alguna de sus partes, frescas, conservadas o preparadas, sin extracción de sus componentes, y de utilización habitual en nuestra dieta. El consumo de semillas de hortalizas es a través de germinados de hortalizas, actualmente se realiza normalmente en crudo, aportando beneficios y compuestos que promueven la salud.

Dentro de los compuestos bioactivos presentes en las hortalizas podemos encontrar, la melatonina que ha sido detectada en cebolla (Hattori y col., 1995); y los compuestos fenólicos detectados en esta hortaliza, principalmente flavonoides (quercetina) (Marotti y Piccaglia, 2002), mientras que en el brócoli destacan los flavonoles (glucósidos de quercetina y glucósidos de kaempferol) y ácidos hidroxicinámicos (Vallejo y col., 2003); por otro lado, en la col roja destaca el contenido de lignanos, así como de flavonoides (quercetina) (Chun y col., 2004; Kuhnle y col., 2007), mientras que en el rabanito destaca el contenido de flavonoles (kaempferol) y lignanos(Lugast y Hovari, 2000; Peñalvo y col., 2007).

Además de los compuestos bioactivos mencionados anteriormente, varios estudios han demostrado que los brotes de crucíferas como el brócoli (*Brassica oleracea L. var. Italica*) y el rábano (*Raphanus sativus*) son muy ricas en fitoquímicos que promueven la salud constituyentes tales como los glucosinolatos relacionados con la prevención del cáncer además de tener propiedades antioxidantes (Tian y col., 2005; Barillari y col., 2005). Un estudio realizado *in vivo* mostró que germinados de rabanito son capaces de reducir la hiperglucemia en la diabetes y además, son eficaces en su prevención primaria en modelos animales (Taniguchi y col., 2006). Por otro lado, el consumo de hortalizas está relacionado con un descenso del riesgo de padecer enfermedades coronarias y una reducción de la presión sanguínea (He y col., 2007).

Por lo tanto, la utilización del proceso de germinación puede ser una estrategia muy adecuada para conseguir alimentos con características nutricionales y sensoriales mejoradas e incrementar los niveles de compuestos bioactivos de carácter antioxidante. La efectividad de la germinación para mejorar el valor nutricional así como la biodisponibilidad de antioxidantes de la dieta, concretamente, de la melatonina en semillas de legumbres y hortalizas no ha sido evaluada hasta la fecha.

3. Melatonina

La dieta mediterránea aporta una serie de metabolitos secundarios de las plantas, que ejerce propiedades beneficiosas sobre la salud denominados compuestos bioactivos. Desde el punto de vista fitoquímico, se pueden agrupar en tres grupos: compuestos fenólicos, isoprenoides y alcaloides. Dentro de esta Memoria sólo destacaremos el papel de los alcaloides, concretamente el compuesto indólico de melatonina.

Los alcaloides comprenden una serie de metabolitos muy diversos entre los que se encuentran los compuestos indólicos, tales como las indolaminas, entre ellas destaca la melatonina (Iriti y col., 2010). Aislada por primera vez a partir de glándulas pineales bovinas (Lerner y col., 1958), es una hormona indólica, derivada del triptófano, que está implicada en muchos procesos fisiológicos.

3.1. Estructura y métodos de análisis

La melatonina (N-acetil-5-metoxitriptamina) es una indolamina constituida por un anillo heterociclo indol y dos cadenas laterales, un grupo metoxilo situado en la posición 5 y un grupo amida situado en la posición 3 (Figura 3). Su fórmula empírica es $C_{13}H_{16}N_2O_2$, peso molecular 232,27 g/mol, densidad 1,27 g/cc y punto de fusión 116-118º C.

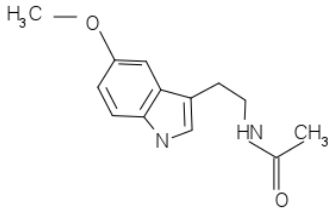


Figura 3. Estructura química de la melatonina.

Su carácter tanto lipofílico (Reiter, 1991) como hidrofílico (Shida y col., 1994) la hace soluble en etanol y en agua, y menos soluble en otros disolventes orgánicos (Garcia-Parrilla y col., 2009). Es una molécula de color amarillo pálido, muy sensible a la luz, cuyo espectro de absorción y emisión se localiza en la región UV con un máximo de 278 nm y 340 nm, respectivamente (Ramírez-Gutiérrez y col., 2009).

El análisis de la melatonina en los alimentos presenta bastantes dificultades debido a las características de la molécula. En primer lugar, el contenido de melatonina en algunas plantas es del orden de µg/g, aunque en otras es mucho menor (ng/g o pg/g), por lo tanto, cualquier método analítico debe ser sensible a pequeñas variaciones. En segundo lugar, el carácter anfipático de la molécula hace difícil la elección de un disolvente que dé lugar a una recuperación completa y a resultados precisos. En tercer lugar, la melatonina es un potente antioxidante y reacciona rápidamente con otros constituyentes de los alimentos por lo que será siempre un requisito fundamental una manipulación cuidadosa de la muestra (Tan y col., 2007a). Por consiguiente, el método de análisis debe tener en cuenta estas limitaciones en un tejido vegetal, mediante el uso de una matriz adecuada, y mostrando sensibilidad y especificidad suficiente.

En primer lugar, debido a las interferencias esperadas con la matriz, resulta imprescindible una etapa previa de extracción y purificación de las muestras, tal como ponen de manifiesto los procedimientos publicados. Diversos estudios han comparado distintos disolventes y diferentes procedimientos de extracción, con el objetivo de determinar cuál es el más adecuado para una extracción eficaz de la melatonina (Arnao y Hernández-Ruiz, 2009a). Los pre-tratamientos descritos para la purificación de las muestras, antes de la medición cromatográfica, incluyen la extracción asistida por

ultrasonido (Cao y col., 2006), la extracción líquido-líquido (Reiter y col., 2005) y la extracción en fase sólida (SPE)(Garcia-Parrilla y col., 2009). La extracción en fase sólida es una técnica de limpieza de muestras simple, rápida y económica, por lo que permite la preconcentración de la muestra con un riesgo mínimo de pérdida o contaminación de la misma. Una columna SPE consiste en un lecho adsorbente de partículas gruesas (C18) mantenido en el interior de un tubo desechable. El fundamento de la técnica consiste en que el componente de interés resultará retenido en la fase sólida mientras que los contaminantes de la matriz serán eluidos.

Diferentes métodos analíticos se han utilizado, hasta la fecha, para cuantificar melatonina en los alimentos. Entre ellos se incluyen técnicas cromatográficas como la cromatografía líquida de alta eficacia acoplada a detector de fluorescencia (HPLC-FL) (Pothinuch y Tongchitpakdee, 2011; Mercolini y col., 2012; Setyaningsih y col., 2012) o espectrómetro de masas (HPLC-MS) (Cao y col., 2006; Vitalini y col., 2011; Kocadağlı y col., 2014), cromatografía gaseosa acoplada a espectrometría de masas (CG-MS)(Van Tassel y col., 2001; González-Gómez y col., 2009), técnicas inmunológicas como el radioinmunoensayo (RIA), enzimoinmunoensayo (EIA) (Iriti y col., 2006; de la Puerta y col., 2007) e inmunoprecipitación (Harumi y Matsushima, 2000; Pape y Lüning, 2006), y otras técnicas como la quimioluminiscencia y la espectrofotometría (Garcia-Parrilla y col., 2009).

Las técnicas que presentan una mayor sensibilidad y especificidad para la cuantificación de melatonina son las cromatográficas. La aplicación de **cromatografía líquida de alta eficacia (HPLC)** con un detector de fluorescencia es el método más utilizado en una gran variedad de matrices heterogéneas, alcanzando límites de detección de hasta 3 pg/mg (Vitale y col., 1996). Debido a que la melatonina posee fluorescencia cuando es irradiada a ciertas longitudes de onda, se obtiene un buen resultado con este detector. Las condiciones de detección se han fijado a una longitud de onda de excitación de 285 nm y de emisión de 345 nm. El tipo de columna cromatográfica utilizada en la mayoría de las determinaciones se trata de una C18 acoplada con una precolumna del mismo material. Se han empleado distintas combinaciones de disolventes para la fase móvil obteniendo adecuados resultados cuando se emplea agua: metanol (60:40)(Arnao y Hernández-Ruiz, 2007), tampón de fosfato potásico y acetonitrilo (20%) (Reiter y col., 2005), ácido fórmico (0,1%) y metanol (40:60) (Rodríguez-Naranjo y col., 2011; Stürtz y col., 2011). Asimismo, diferentes autores utilizan junto a la cromatografía líquida de alta eficacia, la espectrometría de masas (HPLC-MS) en la determinación de melatonina para aportar una mayor información estructural, aumentando así la validación de los resultados (Hernández-Ruiz y Arnao, 2008; Stürtz y col., 2011). En algunas ocasiones, el detector de masas se ha equipado con un sistema de ionización por electrospray (ESI) para

obtener resultados más precisos, concretamente en vinos (Rodríguez-Naranjo y col., 2011).

Dentro de la **cromatografía de gases**, la más empleada es la cromatografía gases/masas (CG-MS) que proporciona un método alternativo para una determinación específica, y en algunos ensayos se han podido comprobar mejores niveles de sensibilidad que con HPLC, siendo su uso menor debido al mayor coste y mantenimiento del equipo cromatográfico (Van Tassel y col., 2001).

Las **técnicas inmunológicas** se han utilizado con éxito para cuantificar la melatonina en las matrices biológicas, así como en matrices vegetales. Estas son el radioinmunoensayo (RIA) y el inmunoensayo enzimático (ELISA). El RIA es una técnica sensible y específica basada en la reacción antígeno-anticuerpo de tipo competitivo, el anticuerpo específico (Ac), reacciona con el correspondiente antígeno (Ag*) marcado radiactivamente y se produce la competición entre el antígeno marcado y el no marcado (el que se quiere determinar) por la unión al anticuerpo, por lo tanto, cuanto mayor sea la radiactividad detectada menor cantidad hay del antígeno a determinar y viceversa. El ELISA es una técnica basada en la reacción antígeno-anticuerpo, se utiliza una enzima de unión al antígeno (Ag) o al anticuerpo (Ac), covalentemente y de forma específica (puentes de hidrógeno, enlaces iónicos, interacciones hidrófobas y fuerzas de van der Waals). Al estar uno de los componentes (antígeno o anticuerpo) marcado con una enzima e insolubilizado sobre un soporte (inmunoadsorbente) la reacción antígeno-anticuerpo queda inmovilizada y, por tanto, mediante la adición de un substrato específico que genera el producto coloreado cuantificable, de tal forma que a mayor concentración de melatonina menor producto coloreado. Para la determinación de melatonina en alimentos se han empleado kits comerciales basados en el ensayo ELISA de tipo competitivo (IBL-Hamburg), como por ejemplo en aceite de oliva (de la Puerta y col., 2007), extractos de piel de uva (Iriti y col., 2006), vino(Iriti, 2009; Rodríguez-Naranjo y col., 2011), en leche de vaca (Kollmann y col., 2008) y en plantas medicinales (EK-DSM, BuhlmannLaboratories-Suiza) (Ansari y col., 2010).

La **quimioluminiscencia** se ha propuesto como otra técnica para la determinación de melatonina (Lu y col., 2002; Harasimowicz y col., 2012). El principio del ensayo se basa en la quimioluminiscencia que se emite después de la reacción de la melatonina con H_2O_2 y acetonitrilo bajo condiciones alcalinas. La formación de oxígeno singlete en la reacción implica que otros compuestos además de la melatonina, como los terpenos y los compuestos fenólicos, entre otros, pueden interferir en el análisis. Por este motivo este método no se recomienda en frutas y verduras.

También se puede determinar espectrofotométricamente, utilizando el $KMnO_4$ y el formaldehído en medio ácido, sin embargo, este método es poco sensible debido a las bajas concentraciones en las que se encuentra la melatonina en alimentos, aunque, se ha utilizado en los extractos de piel de uva (Iriti y col., 2006).

3.2. Contenido de melatonina en la dieta

Esta indolamina se encuentra en muchos órganos de las plantas como raíces, tallos, hojas, flores, frutas y semillas en un amplio rango de concentraciones. Cabe señalar que, de acuerdo con el carácter antioxidante de la melatonina, se espera encontrar mayores cantidades en las semillas, por su posible papel protector frente al estrés oxidativo durante la germinación, aunque existe una amplia variación entre las diferentes matrices vegetales (Paredes y col., 2009; Posmyk y Janas, 2009). Además de las semillas, las frutas se consideran otra fuente natural de melatonina que, en función de los hábitos alimentarios, pueden representar una contribución significativa a la dieta. De hecho, la melatonina está presente en las fresas, kiwis, piñas, plátanos y manzanas con niveles que oscilan entre 0,012 ng/g y 0,048ng/g de materia fresca, destacando las cerezas con contenidos de melatonina de 14 ng/g de materia fresca (Tabla 2). Cabe destacar el consumo de frutas que contienen ácido ascórbico, parece proteger a la melatonina de la oxidación (Dubbels y col., 1995; Reiter y col., 2007).

Tabla 2. Contenido de melatonina en frutas.

NOMBRE CIENTÍFICO	NOMBRE COMÚN	TEJIDO	MÉTODO ANALÍTICO	Melatonina (ng g^{-1})	REFERENCIA
Actinidia chinensis	Kiwi	Fruta	RIA, HPLC-FL	0,024	(Hattori y col., 1995)
***Ananas comosus* L.**	Piña	Fruta	RIA, HPLC-FL	0,036	(Hattori y col., 1995)
***Musa paradisiaca* L.**	Banana	Fruta	RIA, CG-MS	0,047	(Dubbels y col., 1995)
Malus domestica	Manzana	Fruta	RIA, HPLC-FL	0,048	(Hattori y col., 1995)
Prunus cerasus	Cereza	Fruta	HPLC-ECD	13,460	(Burkhardt y col., 2001)
Fragaria ananassa	Fresa	Fruta	RIA, HPLC-FL	0,012	(Hattori y col., 1995)
Fragaria ananassa	Fresa	Fruta	LC-MS	1,38-11,26	(Stürtz y col., 2011)
Vitis vinifera	Uva	Piel	ELISA, HPLC-FL	0,005-0,965	(Iriti y col., 2006)
Olea europaea	Oliva	Hojas	HPLC, ELISA	4,3	(Zohar y col., 2011)
Olea europaea	Oliva	Pulpa	HPLC, ELISA	0,532	(Zohar y col., 2011)
Morus spp.	Morera	Hojas	HPLC, ELISA	0,99	(Zohar y col., 2011)
Ficus carica	Higuera	Hojas	HPLC, ELISA	12,915	(Zohar y col., 2011)
Ficus carica	Higuera	Fruta	HPLC, ELISA	3,959	(Zohar y col., 2011)

Fuente: Elaboración propia.

Por otro lado, se ha detectado melatonina en hortalizas (tomate, pepino y cebolla) con niveles de 0,001 a 0,032 ng/g (Dubbels y col., 1995; Hattori y col., 1995). Se han observado que partes comestibles subterráneas, contienen cantidades de melatonina superiores como las zanahorias (0,055 ng/g de materia fresca). Asimismo, destaca el contenido en cereales (avena, cebada, arroz y maíz) desde 0,378 a 1,796 ng/g (Hattori y col., 1995). También, se han detectado elevados contenidos en leguminosas como el altramuz, 16-18 ng/g (Arnao y Hernández-Ruiz, 2007) (Tabla 3).

Tabla 3. Contenido de melatonina en hortalizas, cereales, frutos secos (F.S.) y legumbres.

	NOMBRE CIENTÍFICO	NOMBRE COMÚN	TEJIDO	MÉTODO ANALÍTICO	Melatonina (ng g^{-1})	REFERENCIA
Hortalizas	***Allium cepa* L.**	Cebolla	Bulbo	RIA, HPLC-FL	0,032	(Hattori y col., 1995)
	Beta vulgaris	Remolacha	Raíz	RIA, LC-MS	0,001	(Dubbels y col., 1995)
	Spinacia oleracea	Espinaca	Hoja	RIA, HPLC-FL	0,039	(Hattori y col., 1995)
	Chenopodium rubrum	Cenizo rojo	Brotes	RIA, LC-MS/ MS	0,240	(Kolár y col., 1997)
	Daucus carota	Zanahoria	Raíz	RIA, HPLC-FL	0,055	(Hattori y col., 1995)
	Apium graveolens	Apio	Semilla	HPLC-ECD	7	(Manchester y col., 2000)
	Brassica oleracea	Col	Hoja	RIA, HPLC-FL	0,107	(Hattori y col., 1995)
	Raphanus sativus	Rábano	Raíz	RIA, HPLC-FL	0,657	(Hattori y col., 1995)
	Cucumis sativus	Pepino	Fruta	RIA, HPLC-FL	0,025	(Hattori y col., 1995)
	Cucumis sativus	Pepino	Fruta	RIA, LC-MS	0,005	(Dubbels y col., 1995)
	Asparagus aphyllus	Espárrago	Hojas	HPLC, ELISA	0,142	(Zohar y col., 2011)
	***Asparagus officinalis* L.**	Espárrago	Brotes	RIA, HPLC-FL	0,010	(Hattori y col., 1995)
	Licopersium esculentum	Tomate	Fruta	RIA, LC-MS	0,005	(Dubbels y col., 1995)
		Tomate	Fruta	RIA, HPLC-FL	0,0322	(Hattori y col., 1995)
Cereales	***Avena sativa* L.**	Avena	Semillas	RIA, HPLC-FL	1,796	(Hattori y col., 1995);
	***Hordeum vulgare* L.**	Cebada	Semillas	RIA, HPLC-FL	0,3781	(Hattori y col., 1995)
	***Oryza sativa* L.**	Arroz	Semillas	RIA, HPLC-FL	1,006	(Hattori y col., 1995)
	***Zea mays* L.**	Maíz	Semilla	RIA, HPLC-FL	2	(Hattori y col., 1995)
F.S.	***Prunus amygdolus***	Almendra	Semilla	RIA, HPLC-ECD	39	(Manchester y col., 2000)
	***Juglans regia* L.**	Nuez	Semilla	HPLC-ECD	3,5	(Reiter y col., 2005)
Legumbres	***Medicago sativum***	Alfalfa	Semilla	HPLC-ECD	16	(Manchester y col., 2000)
	Lupinus albus	Altramuz	Raíz	HPLC-FL	16,2-18,4	(Arnao y Hernández-Ruiz, 2007)
	Glycine max	Soja			4,4	(Zielinski y col., 2001)
	Lens sculenta	Lenteja			1,2	(Zielinski y col., 2001)

Fuente: Elaboración propia.

Además, se ha determinado la cantidad de melatonina en plantas medicinales (Tabla 4) como la artemisa (Murch y col., 1997), hipérico (Murch y col., 2000), hinojo, anís (Manchester y col., 2000), y en hierbas medicinales chinas, donde los niveles de melatonina son considerablemente altos, pudiendo alcanzar hasta 3771 ng/g en el caso del Chan tui (*Periostracum cicadae*) o como en la planta de aloe vera (516 ng/g). Estas plantas son conocidas por sus propiedades antioxidantes, y su elevado contenido de melatonina puede potenciar sus efectos farmacológicos (Chen y col., 2003).

Tabla 4. Contenido de melatonina en plantas medicinales.

NOMBRE CIENTÍFICO	NOMBRE COMÚN	TEJIDO	MÉTODO ANALÍTICO	Melatonina (ng g^{-1})	REFERENCIA
Pimpinella anisum	Anís	Semilla	HPLC-ECD	7	(Manchester y col., 2000)
Foeniculum vulgare	Hinojo	Semilla	HPLC-ECD	28	(Manchester y col., 2000)
Tanacetum parthenium	Artemisa	Hojas verdes	HPLC-ECD, RIA	2450	(Murch y col., 1997)
Tanacetum parthenium	Artemisa	Hojas doradas	HPLC-ECD, RIA	1920	(Murch y col., 1997)
Ephedra campylopoda	Efedrina	Hojas	HPLC, ELISA	0,178	(Zohar y col., 2011)
Ephedra campylopoda	Efedrina	Semilla	HPLC, ELISA	0,379	(Zohar y col., 2011)
Trigonella foenum-graecum	Alholva o fenogreco	Semilla	HPLC-ECD	43	(Manchester y col., 2000)
Hypericum perforatum	Hipérico	Hojas	HPLC-ECD, LC-MS/MS	1750	(Murch y col., 1997)
Hypericum perforatum	Hipérico	Flores	HPLC-ECD, LC-MS/MS	4390	(Murch y col., 1997)
***Aloe vera* L.**	Aloe vera	Hojas	HPLC-FL	516	(Chen y col., 2003)
Periostracum cicadae	Chan tui		HPLC-FL	3771	(Chen y col., 2003)
Laurus nobilis	Laurel	Hojas	HPLC, ELISA	8,331	(Zohar y col., 2011)
Laurus nobilis	Laurel	Fruto	HPLC, ELISA	3,71	(Zohar y col., 2011)
Laurus nobilis	Laurel	Semilla	HPLC, ELISA	6,06	(Zohar y col., 2011)
Laurus nobilis	Laurel	Pulpa	HPLC, ELISA	1,82	(Zohar y col., 2011)
Elettaria cardamomum* L. *Maton	Cardamomo verde	Semilla	HPLC-ECD	12	(Manchester y col., 2000)
Brassica nigra	Mostaza negra	Semilla	HPLC-ECD	129	(Manchester y col., 2000)
Sinapis alba	Mostaza blanca	Semilla	HPLC-ECD	189	(Manchester y col., 2000)
Coffe canephora	Café robusta	Granos	HPLC/ESI-MS	5800-8000	(Ramakrishna y col., 2012)
Coffe arabica	Café arábica	Granos	HPLC/ESI-MS	6800-9600	(Ramakrishna y col., 2012)

Fuente: Elaboración propia.

En la actualidad debido al gran interés que suscita esta neurohormona, se estudia el efecto del procesamiento de alimentos en los niveles de melatonina (Tabla 5) (de la Puerta y col., 2007; Guerrero y col., 2007; Maldonado y col., 2009; Rodríguez-Naranjo y col., 2011; Pothinuch y Tongchitpakdee, 2011) por ello se ha estudiado el contenido de melatonina en algunos alimentos procesados como son el aceite de oliva, el vino, la cerveza y zumos (de la Puerta y col., 2007; Maldonado y col., 2009; Rodríguez-Naranjo y col., 2011; Mena y col., 2012).

Actualmente, la evidencia de que la melatonina es un compuesto bioactivo presente de forma natural en los alimentos, está suficientemente demostrada. Sin embargo, existe escasa literatura acerca de los niveles de melatonina en diferentes matrices alimentarias o de los métodos analíticos más adecuados para su cuantificación, así como los posibles efectos beneficiosos para la salud de su ingestión a través de la dieta.

Tabla 5. Contenido de melatonina en alimentos procesados.

Alimento		MÉTODO ANALÍTICO	Melatonina (ng mL^{-1})	REFERENCIA
Vinos	Vino tinto (Italia)	HPLC-FL	0,5	(Mercolini y col., 2012)
	Vino tinto	EC	0,3	(Stege y col., 2010)
	Vino tinto (variedades)	HLPC-MS/MS	74-424	(Rodríguez-Naranjo y col., 2011)
	Vino blanco (Italia)	HPLC-FL	0,4	(Mercolini y col., 2012)
	Vino blanco	EC	0,16	(Stege y col., 2010)
	Vino blanco	HLPC-MS/MS	390,82	(Rodríguez-Naranjo y col., 2011)
Cerveza	Cervezas comerciales	ELISA	0,52-1,70	(Maldonado y col., 2009)
Aceite de oliva	Virgen extra D.O.	ELISA	0,71-1,19	(de la Puerta y col., 2007)
	Refinado	ELISA	0,53-0,75	(de la Puerta y col., 2007)
Aceite de girasol	Refinado	ELISA	0,50	(de la Puerta y col., 2007)

Fuente: Elaboración propia.

En base a los estudios llevados a cabo, se ha postulado que la melatonina puede ser un importante componente bioactivo de ciertos estilos dietéticos, tales como la dieta mediterránea (Iriti y col., 2010). La ingestión de productos alimenticios que contienen melatonina puede contribuir a aumentar los niveles séricos de este compuesto en animales, incluyendo el ser humano (Maldonado y col., 2009; Garcia-Parrilla y col., 2009). Asimismo, se ha comprobado que una dieta con alimentos ricos en melatonina contribuye a aumentar la capacidad antioxidante del organismo, reduciendo la oxidación producida por los lípidos en el estómago, lo que ayuda a preservar la integridad de la mucosa gastrointestinal y protege contra la formación de úlceras

gástricas provocadas por el estrés (Brzozowski y col., 2005). Por otra parte, no se ha observado ningún efecto tóxico o adverso de la administración diaria de melatonina (10 a 300 mg) (Sánchez-Barceló y col., 2010), lo que puede alentar la administración a largo plazo en pacientes que sufren determinadas enfermedades; aunque son incompletos los estudios acerca de su absorción y biodisponibilidad (Reiter y col., 2005).

3.3. Biosíntesis de melatonina

3.3.1. En vegetales

La presencia de la melatonina en las plantas se detectó por primera vez en 1995 (Van Tassel y col., 1995; Dubbels y col., 1995; Hattori y col., 1995), desde entonces se han realizado numerosos estudios en plantas monocotiledóneas como en dicotiledóneas, incluyendo frutas, verduras, legumbres, cereales, semillas comestibles y hierbas medicinales (Murch y col., 1997; Manchester y col., 2000; Chen y col., 2003; Kolár y Macháckova, 2005).

Las plantas son capaces de sintetizar melatonina sin embargo presenta algunas diferencias respecto a la biosíntesis en animales (Figura 4). En las plantas, la síntesis de serotonina es diferente, la primera reacción está catalizada por la acción de la triptófano descarboxilasa (TDC) que a partir de triptófano da lugar a triptamina, a continuación se produce la hidrólisis de triptamina a triptamina 5-hidroxilasa para dar lugar a la serotonina, mediante la triptófano hidroxilasa (T5H). Se ha observado que en las plantas el L-triptófano no sólo es precursor de serotonina, sino también del ácido indolacético (IAA) y de otras auxinas como por ejemplo indol-3 butírico (IBA) y ácido p-hidroxifenilacético. El IAA se puede sintetizar a través de tres vías diferentes, como se muestra en la imagen, primeramente a parir del ácido indol 3-piruvico (IPA), transformándose a continuación a ácido 3-indol acetaldehído (IAAId), en segundo lugar mediante indol 3-acetamida (IAM), y finalmente mediante indol 3-acetonitrilo (IAN). Alternativamente, el IAA puede también ser sintetizado a partir de triptamina catalizada por triptamina desaminasa (TDA) y este intermedio está directamente relacionado con la serotonina y la biosíntesis de melatonina. La vía dominante es la de triptófano a través del compuesto 5-hidroxitriptófano que da lugar a la serotonina y de N-acetilserotonina mediado por la enzima Serotonin N-acetiltransferasa (SNAT) y convertido a melatonina por la hidroxiindol metiltransferasa (HIOMT) mediante la ASNMT (acetilserotonina N-metiltransferasa) como donante grupo metilo. Sin embargo, a una cierta velocidad, la secuencia alternativa, serotonina a través de 5-metoxitriptamina a melatonina también es posible (Posmyk y Janas, 2009; Tan y col., 2012).

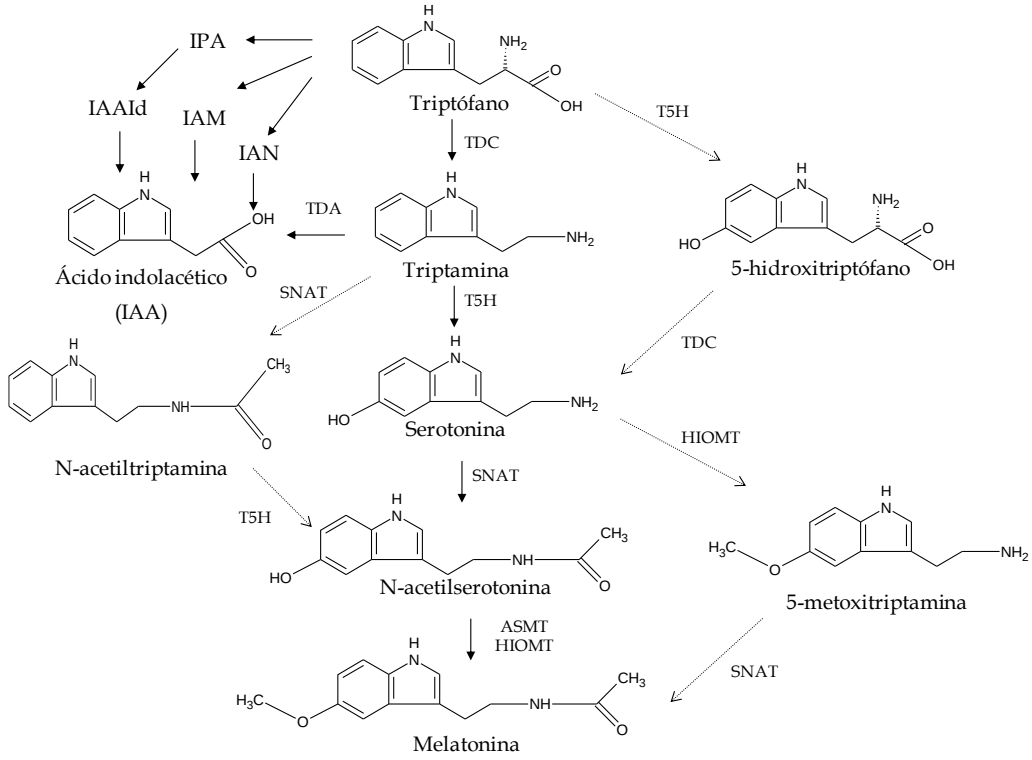


Figura 4. Síntesis de melatonina en plantas.
Las flechas discontinuas representan reacciones alternativas descritas en casos particulares.
Fuente: (Posmyk y Janas, 2009), modificado.

La presencia en plantas indica que también realiza diversas funciones (Figura 5). En base a los relevantes niveles detectados en las plantas, se ha sugerido que la melatonina puede desempeñar un papel importante en el sistema de defensa antioxidante de la planta, implicada en la protección contra factores ambientales adversos, como la sequía, la temperatura, etc y procesos endógenos que generan radicales libres. Asimismo, retrasa la senescencia de las hojas, ya que existen numerosos estudios que revelan este carácter protector cuando se aplican exógenamente agentes estresantes a las plantas (Arnao y Hernández-Ruiz, 2009a). Al igual que en animales, se ha relacionado con la regulación de los ritmos circadianos de las plantas, existe una evidencia de una oscilación similar diurna, afectando al ritmo bioluminscente y el fotoperiodo. También está relacionada con el crecimiento y desarrollo de las plantas, debido a su similitud estructural con la auxina (ácido indolacético, IAA), ya que ambos son derivados indólicos y es posible que realicen efectos similares (Chen y col., 2009). Las plantas además, pueden ser capaces de

absorber la melatonina del suelo o del medio de cultivo, a través de las raíces y transportarla, ya que muchos microorganismos contienen melatonina y su descomposición puede dar lugar a dicho compuesto (Paredes y col., 2009).

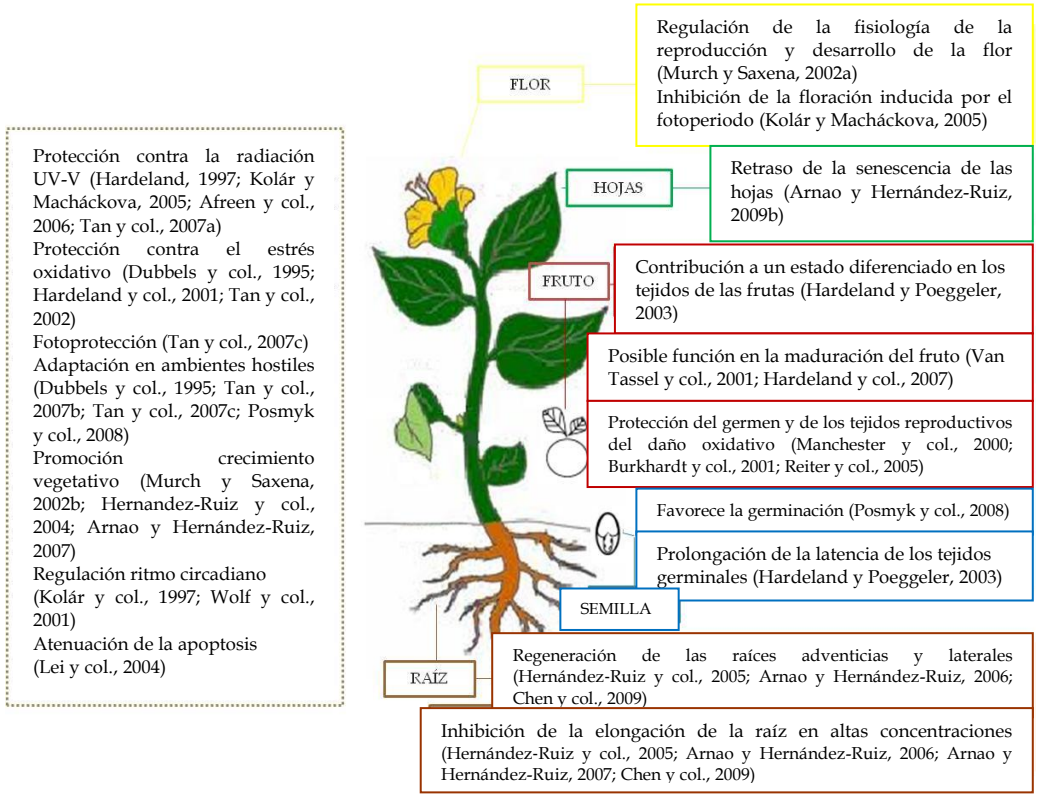


Figura 5. Funciones de melatonina en plantas.
Fuente: (Paredes y col., 2009).

3.3.2. En humanos

La biosíntesis de la melatonina en los animales se inicia por la absorción del triptófano, aminoácido esencial, en las células del parénquima pineal, situado en la glándula pineal (en el cerebro). Está formada por células neuroepiteliales, entre las cuales se encuentran las células gliales y pinealocitos, produce la melatonina. La actividad de la glándula pineal depende de las condiciones lumínicas, la luz inhibe la producción y la oscuridad la aumenta, siendo la retina el punto de reconocimiento del estado lumínico y desde donde se emite la información luminosa hasta la glándula pineal.

La síntesis de melatonina en humanos se realiza tal y como se resume en la Figura 6.

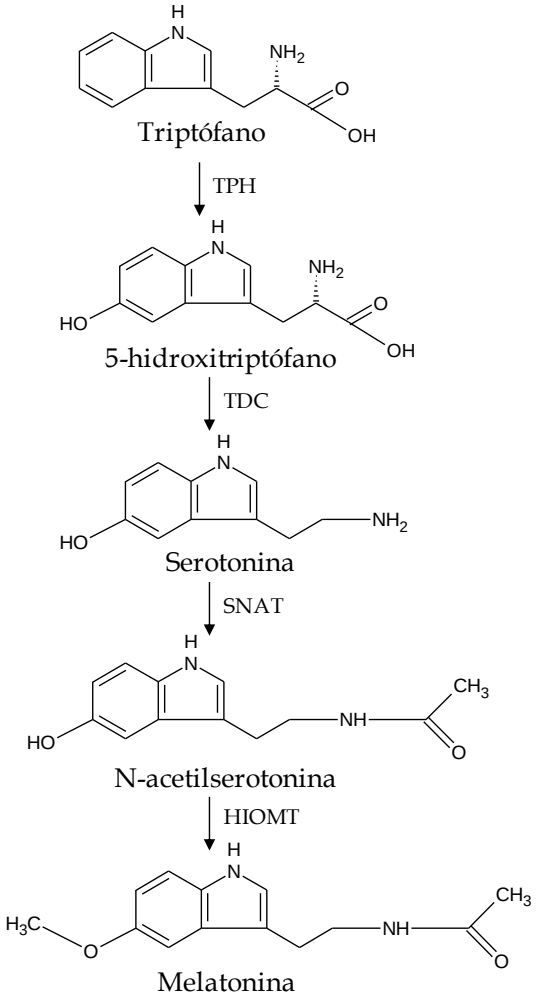


Figura 6. Síntesis de melatonina en humanos.
Fuente: (Fernández-Montesinos y col., 2010), modificado.

Una vez producida, la melatonina pasa a la circulación sanguínea alcanzando una concentración de 0,5 nM como máximo, durante su acrofase (de 2:00 a 3:00) y es transportada en el plasma, parte unido a la albúmina (70%) y parte en forma libre (30%) que pasa a la saliva (Escames y Acuña-Castroviejo, 2009). La producción endógena normal se sitúa entre 28 y 30 µg/día (Lane y Moss, 1985; Short, 1993), alcanza unas concentraciones altas durante la noche, los valores en plasma oscilan entre 80 y 200 pg/mL dependiendo del individuo (Reiter, 1992) y concentraciones basales durante el día. Su vida media oscila entre 20 y 90 minutos (Illnerova y col., 1979; Vakkuri y col., 1985).

La melatonina, también se sintetiza en otras partes del cuerpo como el tracto gastrointestinal (Bubenik, 2002), la retina (Gern y Ralph, 1979), páncreas, ovarios, testículos, piel y medula ósea (Huether y col., 1992; Huether, 1993). Sin embargo, la melatonina sintetizada fuera de la glándula pineal escasamente pasa a la circulación, funciona localmente como una señal autocrina o paracrina (Pandi-Perumal y col., 2006). Por este motivo, los niveles de melatonina tisulares son cientos de órdenes de magnitud mayores que los de plasma (Reiter y Tan, 2003). Ello refuerza la teoría de que la melatonina realice otras funciones además de la hormonal. A nivel gastrointestinal, la melatonina se produce en las células enterocromafines, pero también se puede obtener melatonina del plasma o del tubo digestivo. Aunque las concentraciones se mantienen moderadas, el tracto gastrointestinal debido a su tamaño puede contener hasta 400-500 veces más de melatonina que la glándula pineal (Bubenik, 2002; Hardeland y Pandi-Perumal, 2005). También se encuentra en altas concentraciones en la bilis y en la circulación enterohepática (Tan y col., 1999; Bubenik y col., 1999).

3.4. Metabolismo

Esta molécula se metaboliza principalmente en el hígado, donde se transforma en un compuesto hidrosoluble, la 6-hidroximelatonina (6-HMEL), por la acción de las enzimas del citocromo P450 (CYP1A1, CYP1A2 y CYP1B1) tras la conjugación con anión sulfato o ácido glucurónico, fácilmente excretada por la orina (95%) siendo el metabolito mayoritario en humanos el 6-sulfatoximelatonina (aMTs6). La enzima CYP2C1, también en el hígado, mediante o-dimetilación de la melatonina produce el compuesto N-acetilserotonina (NAS) representa el metabolito minoritario en humanos. Otro metabolito generado es la 3-hidroximelatonina cíclica (C3-OHM), que se forma tras la depuración de dos OH y éste puede ser considerado como un biomarcador de los niveles endógenos de OH (Ma y col., 2006).

En la glándula pineal o en el sistema nervioso central, también se produce la conversión de melatonina en kinureninas mediante ruptura oxidativa del anillo pirrólico, por la acción de la enzima indolamina-2,3-dioxigenasa (IDO) o a través de reacciones no enzimáticas por catálisis a través de oxiferrilhemoglobina, hemina o con interacciones especies reactivas de oxigeno: $\bullet OH$, $\bullet O_2^-$, H_2O_2, radicales orgánicos catiónicos y $\bullet O_2^-$ y O_3. En este caso, la melatonina se transforma en N1-acetil-N2-formil-5-metoxikinuremina (AFMK), como metabolito secundario. Todo esto indica que AFMK es el metabolito central de la oxidación de melatonina en tejidos no hepáticos. Posteriormente por la acción de la arilamina formamidasa, la hemoperoxidasa (catalasa) o la liberación inducida por la luz UV de monóxido de carbono, se transforma en N1-acteil-5-metoxikinuremina (AMK), como metabolito terciario (Hardeland y col., 1993; Tan y col., 2000; Hardeland y col., 2004). Estos

compuestos, metabolitos activos, son metabolizados en tejidos específicos, también se forman en la piel y en otros tejidos periféricos, tanto por vía enzimática como no enzimática. Dos nuevos metabolitos han sido detectados en suero a partir de AMK, siendo O-AMK, como metabolito intermedio, y G-O-AMK, el metabolito final (Niu y col., 2010) (Figura 7).

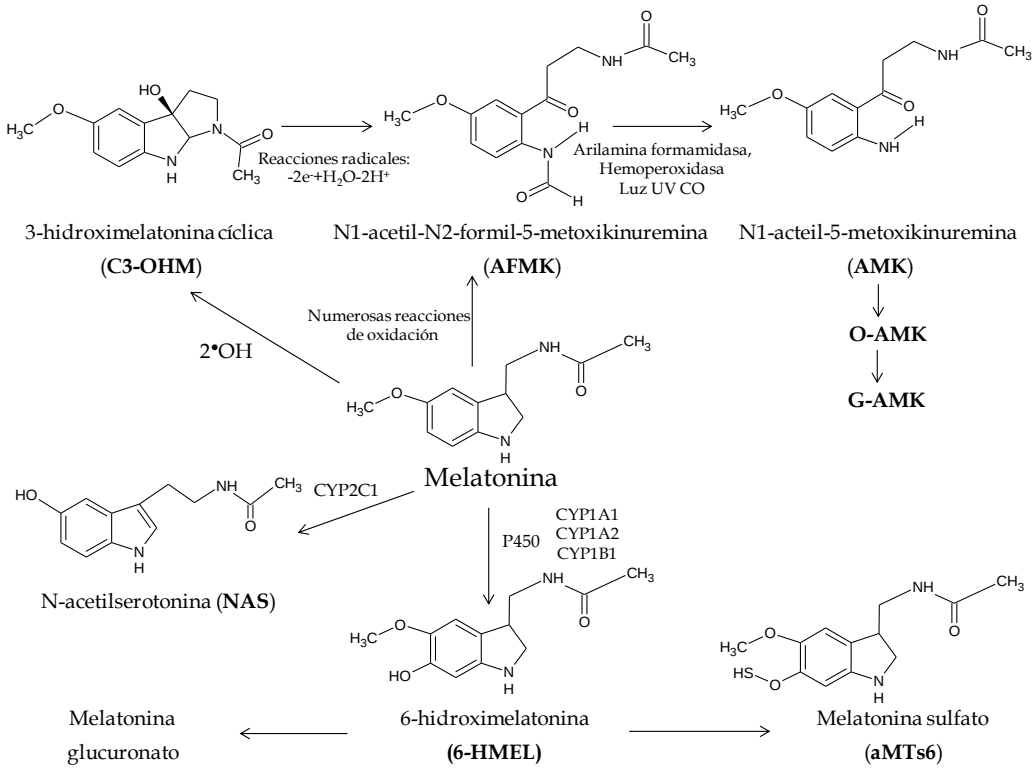


Figura 7. Metabolitos derivados de la melatonina.
Fuente: (Ma y col., 2006), modificado.

Es importante destacar que no solo la melatonina sino sus metabolitos son eficaces en reducir el daño producido por radicales libre, dando lugar a la cascada antioxidante (Hardeland y Pandi-Perumal, 2005).

3.5. Biodisponibilidad

Se conoce que la melatonina se absorbe rápidamente, alcanzando mayores niveles en plasma a los 30 minutos y 2 horas. El tiempo de vida media varía entre 10 y 60 minutos, después de una administración exógena (Peuhkuri y col., 2012). Investigaciones realizadas en animales, en particular en roedores de laboratorio, los

parámetros farmacocinéticos (tiempo de vida media y biodisponibilidad) son uniformes y presentan pequeñas variaciones interindividuales. En comparación con los roedores, la biodisponibilidad de la melatonina en seres humanos es pobre. Un estudio realizado mediante la marcación con deuterio de melatonina utilizada como referencia, mostró que el consumo oral de melatonina fue inferior al 1% observándose diferencias entre ambos sexos, siendo la biodisponibilidad de melatonina en mujeres doble que la mostrada en hombres (Fourtillan y col., 2000). La baja biodisponibilidad de la melatonina parece ser debida a su paso por el hígado; sin embargo, cierta cantidad puede ser degradada por la CYP450 1B1 gastrointestinal. De esta forma, la melatonina puede participar en la circulación entero-hepática o puede ser utilizada por mecanismos no enzimáticos que incluyen una interacción con ROS/RNS. Se han detectado altos niveles de melatonina en bilis de ratas (Tan y col., 1999) y en el tracto biliar de seres humanos (Messner y col., 2001) lo que sugiere que parte del consumo oral puede alcanzar la circulación entero-hepática.

Además de su baja biodisponibilidad, se han observado variaciones individuales, de hasta 37 veces, que pueden ser debidas a la diferente respuesta oral de los individuos tras la ingesta, como consecuencia de las propiedades heterogenéas de expresión de genes del citocromo P450C en seres humanos. Por lo tanto, para obtener un tratamiento adecuado, es preciso realizar la individualización de la dosis, es decir, ajustar la dosis en función de los niveles en plasma o saliva tras la administración de melatonina (Tan y col., 2007a).

La interacción con otros compuestos puede influir en la biodisponibilidad de la melatonina, de manera que cuando ésta se suministra junto a 200 mg cafeína (equivalente a una taza de café), la biodisponibilidad se incrementa hasta el 140%, debido a que ambas moléculas son sustratos del CYP1A2 (Härtter y col., 2003). Además, si se administra melatonina junto a vitaminas E y C, se constata una mayor biodisponibilidad. El conocimiento de la farmacocinética de la melatonina y su interacción con otras sustancias puede ayudar a encontrar la dosis adecuada para cada situación e individuo (Tan y col., 2007a).

Desde que se descubrió la presencia de melatonina en plantas superiores, se han ampliado las perspectivas sobre el estudio de la melatonina exógena como un componente natural de los alimentos. En este sentido, varias especies de plantas/cultivos han sido estudiadas como posibles fuentes de nutracéuticos, y/o suplementos dietéticos, que aporten cantidades significativas de melatonina en la dieta. Existe numerosa bibliografía de estudios clínicos sobre la biodisponibilidad de melatonina pura. También se dispone de investigaciones en animales sobre los efectos de la administración de la melatonina contenida en vegetales y algunos estudios en humanos (Reiter y col., 2005; Reiter y col., 2007; Maldonado y col., 2009) sobre la

biodisponibilidad de melatonina en fuentes naturales, como frutas y frutos secos, tal y como se refleja en la Tabla 6.

Tabla 6. Estudios de biodisponibilidad de melatonina dietética.

Ensayos	Duración	Dosis	Efecto	Referencia
Animales				
Pollos	1,5 h	3 ng /g Mel (maíz, sorgo, frijol y arroz)	Incremento de 10 a 35 pg/mL melatonina en suero	(Hattori y col., 1995)
SD macho	4 h	1 g de nueces (Mel: 3,5-1 ng/g)	Incremento de melatonina en plasma	(Reiter y col., 2005)
Humanos				
12 hombres sanos	72 h	200g de cerezas (*Prunus avium* L.) 2 veces al día	Incremento de 6-sulfatoximelatonina urinaria	(Garrido y col., 2010)
12 hombres sanos	60, 120, 180,y 270 min	Consumo plátano, naranja o piña	Incremento hasta 50-200 pg/mL	(Sae-Teaw y col., 2013)

Leyenda: h: hora, min: minutos; SD: ratas Sprague-Dawley; Mel: Melatonina
Fuente: Elaboración propia.

3.6. Beneficios

Debido a los distintos mecanismos de acción de la melatonina se le atribuyen diversas funciones con efectos muy diferentes, todas ello se describe más detalladamente a continuación.

- Regulación de los ritmos circadianos endógenos y circanuales, debido a la presencia del reloj interno (núcleo supraquiasmático del hipotálamo), que está sincronizado por los niveles lumínicos ambientales percibido por la retina y que extiende su sincronización al resto del organismo mediante la síntesis de melatonina, recíprocamente. Bajo fotoperiodos naturales, la duración del pico nocturno de melatonina es directamente proporcional a la duración del periodo de luz. El fotoperiodo cambia con las estaciones del año, siendo las noches más cortas en verano y más largas en invierno, como consecuencia la producción de melatonina es menor en verano y mayor en invierno. Los ritmos circanuales también regulan la reproducción estacional de distintas especies, así como la alimentación, migración, etc…, estos efectos forman parte de la sincronización que la hormona ejerce sobre las funciones biológicas (Reiter, 1993). La administración exógena de melatonina favorece su resincronización ante cambios ambientales regulando los ritmos de temperatura, el ciclo de sueño y vigilia, el nivel de alerta, entre otros procesos (Turek y col., 2001). La disminución de la temperatura corporal, junto con la máxima sensación de fatiga y

reducción del estado de alerta coincide con el aumento de la concentración de melatonina que circula en el plasma y con la producción de sueño.

La European Food Safety Authority (EFSA) desde 2010 alega que la melatonina alivia los sentimientos subjetivos del jet-lag, para ello la dosis establecida es de 0,5-5 mg y debe tomarse antes de acostarse el primer día del viaje, y en días posteriores. Sin embargo, no se ha establecido una relación de causa-efecto en la alegación sobre la regulación del ciclo sueño-vigilia (EFSA, Panel on Dietetic Products Nutrition and Allergies (NDA), 2010).

- Anti-envejecimiento. Muchos estudios demuestran que la concentración plasmática de melatonina disminuye con la edad. El feto la recibe de la madre a través de la placenta (Escames y Acuña-Castroviejo, 2009), no se produce de forma rítmica hasta los 6 meses de vida extrauterina, cuando madura el sistema foto neuroendocrino. Durante las primeras semanas de vida, la melatonina pasa a través de la leche materna, hasta que comienza la producción rítmica. La producción y secreción de melatonina se mantiene hasta la pubertad, cuando disminuye de manera notable, para estabilizarse de nuevo hasta los 35-40 años. A partir de este momento, la producción decae y hacia los 55-75 años la amplitud del pico nocturno es suficientemente pequeña para no ser detectada, lo que condiciona la pérdida de la capacidad cronobiótica del individuo (Reiter, 1992) (Figura 8). Aunque se desconoce el mecanismo, uno de los numerosos factores que pueden explicarlo es debido a la pérdida de actividad de las enzimas encargadas de la síntesis de melatonina (Handerland y col., 2011).

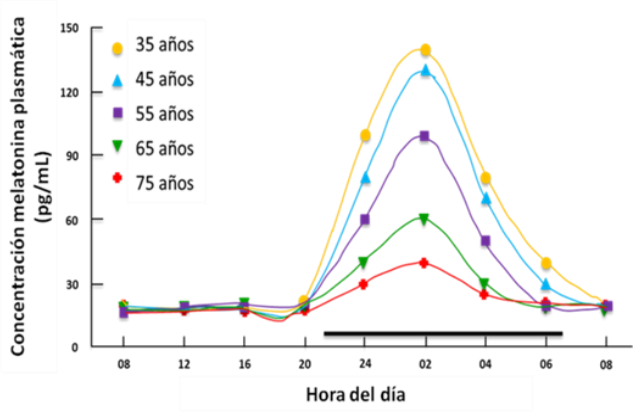


Figura 8. Concentración plasmática de melatonina según la edad.
Fuente: (Escames y Acuña-Castroviejo, 2009).

- Acción inmunomuduladora. La interrelación entre la respuesta inmunitaria y el sistema nervioso, es debido a un eje de regulación neuroendocrino-inmunitario y de receptores para neurotransmisores en algunas células inmunitarias (Dubocovich y Markowska, 2005). El papel inmunomodulador de la melatonina está relacionado con su acción sobre los receptores específicos localizados en las células inmunocompetentes (Maestroni y col., 2002), así como la regulación de la hematopoyesis en la médula ósea (Maestroni, 2001). La administración exógena de melatonina aumenta la producción de anticuerpos (Maestroni y col., 1986) y disminuye las enfermedades autoinmunes (Carrillo-Vico y col., 2005). Asimismo, produce la activación de linfocitos y monocitos/macrófagos, impidiendo el desarrollo de tumores (Martins y col., 1998; Miller y col., 2006).

- Regulación hormonal. La melatonina tiene un papel importante en la regulación del sistema endocrino, destacando la regulación de la hormona liberadora de gonadotropina (GnRH), la estimulación de la secreción de oxitocina, la síntesis de progesterona, la regulación de cortisol y la producción de andrógenos (Schaeffer y Sirotkin, 1995; Balik y col., 2004; Tamura y col., 2008; Tamura y col., 2009).

La melatonina tiene un efecto directo en el aparato reproductor femenino, donde regula la secreción de hormonas esteroideas. En mujeres embarazadas, se incrementa la concentración en plasma y estimula la producción de progesterona, mientras que en mujeres que presentan patrones alterados o niveles reducidos, puede ser la causa de complicaciones como el aborto, pre-eclampsia y la discapacidad neurológica neonatal (Tamura y col., 2008). También se ha detectado mayores niveles de melatonina en suero en mujeres con síndrome del ovario poliquístico, debido a una reducción en la absorción del folículo ovárico; altos niveles de melatonina son esenciales para el crecimiento del folículo, la ovulación y la calidad de los ovoocitos. Mientras que una reducción de las concentraciones de melatonina folicular puede ser la responsable de la anaovulación (Tamura y col., 2009).

- Acción oncostática. La melatonina presenta la capacidad de inhibir la proliferación de células cancerosas, debido a su acción antioxidante y su función inmunomuduladora. La administración de melatonina reduce el crecimiento tumoral y prolonga la supervivencia sobre todo en cánceres dependientes de hormonas reproductoras, como el cáncer mama y de ovario, así como en el cáncer de colon, es decir, aquellos pacientes con enfermedad oncológica presentan menor concentración de melatonina en sangre (Pandi-Perumal y col., 2008).

- Acción vascular. Se ha encontrado que la melatonina es un vasodilatador en la arteria mesentérica y aorta (Girouard y col., 2001) pero, por el contrario, en la arteria caudal actúa como vasoconstrictor (Krause y col., 1995; Ting y col., 1997) dependiendo

de la concentración utilizada, siendo a mayores concentraciones (10^{-10}-10^{-7} M) un agente vasoconstrictor y a menores concentraciones (10^{-7}-10^{-5} M) como vasodilatador (Doolen y col., 1998). Un uso potencial farmacológico de la melatonina puede ser como molécula antihipertensiva ya que reduce la presión arterial sistólica en los seres humanos (Gómez-Moreno y col., 2010; Paulis y col., 2010).

- Acción antioxidante. La melatonina es un importante antioxidante, destacando este aspecto debido a su importancia, esta información se detalla en el siguiente apartado.

- Otras. Numerosos estudios han indicado la importancia de la melatonina en la piel (Slominski y col., 2012), así como el efecto sobre el crecimiento del pelo (Fischer y col., 2008).

3.7. Mecanismo de acción antioxidante

El estrés oxidativo se define como el resultado de un desequilibrio en el organismo entre la producción de especies reactivas de oxigeno (ROS) y los sistemas de defensa antioxidantes, provocando daños a nivel tisular (Sies, 1997) y asociado con el origen de numerosas patologías (Maiese y col., 2010).

Las especies reactivas son entidades químicas que, a bajas concentraciones, participan en la señalización celular; sin embargo, altos niveles tienen efectos nocivos. Se han descrito dos categorías de especies reactivas: los radicales libres y las especies no radicales (Beckman y col., 1994). Los radicales libres tienen uno o más electrones desapareados en su última capa orbital; por este motivo son muy inestables, altamente reactivos y de vida media corta (Halliwell, 2011). Pueden generar reacciones en cadena, transfiriendo electrones a moléculas vecinas, que a su vez pueden convertirse en radicales libres (Ilie y Margina, 2012). Entre las ROS más importantes cabe destacar los aniones superóxido (O_2-), los radicales hidroxilo (·OH), radical alcohoxilo (RO•), radical peroxilo (ROO•). Otras especies reactivas de importancia biológica son las que derivan del nitrógeno (RNS) como el óxido nítrico (NO•), entre otros (Halliwell, 1994). Dentro de las especies no radicales, existen moléculas como el peróxido de hidrógeno (H_2O_2) y el peroxinitrito (ONOO-), que en sentido estricto no son radicales libres, también poseen gran capacidad de reacción y, a altas concentraciones, pueden ocasionar daño oxidativo. Además, existen otras especies reactivas que implican al cloro, hierro, cobre y azufre (Halliwell, 2011).

Además, las ROS ejercen un importante papel preventivo frente a las infecciones ya que constituyen parte del mecanismo de defensa de las células fagocitarias (He y col., 2007), es decir, tienen funciones fisiológicas, pero en exceso pueden producir daño al oxidar diversas moléculas orgánicas. Por esta razón, el organismo dispone de un sistema enzimático antioxidante endógeno que regula de manera natural la oxidación

generada durante los procesos redox celulares, (Sies, 1997; Halliwell, 2006; Veskoukis y col., 2012). En función de su origen los antioxidantes pueden clasificarse en exógenos (provienen de la dieta) y endógenos (sintetizados por el propio individuo). Por otro lado, también se pueden clasificar en antioxidantes enzimáticos y no enzimáticos actúan sistemáticamente para captar radicales libres. Los antioxidantes enzimáticos constan de la superóxido dismutasa (SOD), la catalasa (CAT), la glutatión peroxidasa (GP_X) y glutatión reductasa (GR). Este sistema es la principal defensa frente a los ROS *in vivo* (Figura 9). Pueden catalizar la reacción para descomponer los radicales de anión superóxido en H_2O_2, que será convertido en agua y oxígeno a través de CAT o GPx (Peng y col., 2014).

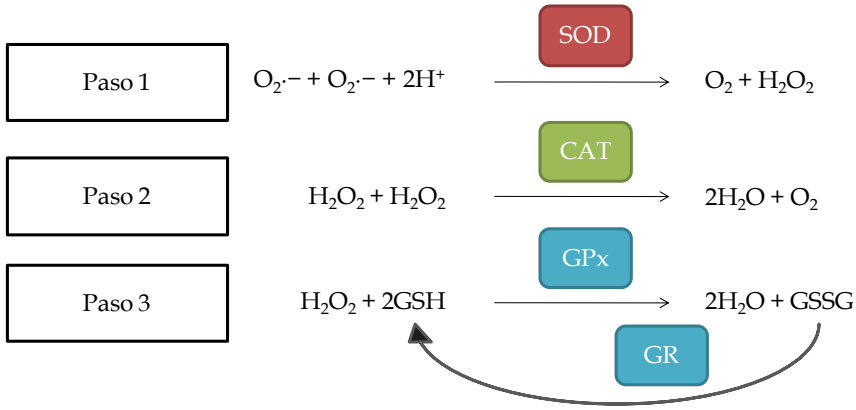


Figura 9. Principales sistemas de defensa antioxidantes enzimáticos *in vivo*.
Fuente: (Peng y col., 2014).

El sistema de defensa antioxidante no enzimático, es el segundo sistema de defensa frente a los radicales libres, no sólo pueden proporcionar una proporción directa contra el daño oxidativo sino que también pueden mejorar de manera sinérgica la función de los antioxidantes endógenos. En este grupo se encuentran el glutatión (GSH) y la coenzima Q10, también las vitaminas C y E, aunque en determinadas circunstancias pueden actuar como prooxidantes (Weinberg y col., 2001). Además de las vitaminas, existen muchas moléculas que actúan como antioxidantes no enzimáticos, tales como los compuestos fenólicos y los carotenoides presentes de forma natural en los alimentos. Estudios epidemiológicos relacionan un alto consumo de frutas y verduras con la prevención de diversas enfermedades, debido a la presencia de estos compuestos (Dueñas y col., 2002); no obstante, algunos autores proponen que los antioxidantes exógenos no generan tanta protección frente al daño oxidativo como los propios antioxidantes endógenos (Gutteridge y Halliwell, 2010).

Los mecanismos de acción de la melatonina son muy diversos (Figura 10), esquemáticamente se pueden dividir en dos, aquellos que están mediados por su unión a un receptor, de membrana o nuclear, y aquellos que se realizan de forma independiente al receptor, como la interacción de proteínas intracelulares y la actividad antioxidante a través de los cuales ejercen sus funciones en mamíferos (Guerrero y col., 2007; Fernández-Montesinos y col., 2010).

La diversidad de respuesta de la melatonina en el cuerpo es atribuida al hecho de que los receptores son expresados en una amplia variedad de tejidos (Witt-Enderby y col., 2003).

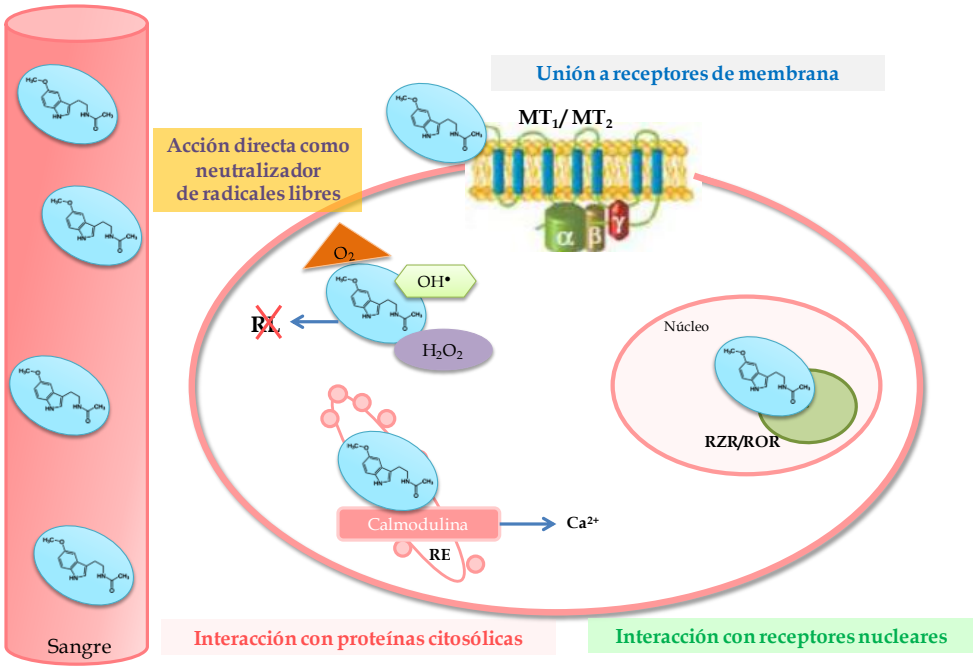


Figura 10. Mecanismo de acción de la melatonina.
Fuente: (Favero y col., 2014), modificado.

En este sentido, la melatonina es un potente antioxidante endógeno, debido a su alta eficacia y papel protector frente a los radicales libres (ROS) y las especies reactivas de nitrógeno (RNS) (Gómez-Moreno y col., 2010). Dentro de los ROS protege frente al radical anión superóxido (O_2),hidroxilo (OH●), alcoxil (RO●), radical peroxilo (ROO●) e hidroperoxil (HOO●) dentro de los RNS incluye el peroxinitrilo (ONOO), óxido nítrico (NO●)y dióxido nitrógeno (NO_2) (Galano y col., 2011).También puede depurar el peróxido de hidrogeno (H_2O_2) y el ácido hipocloroso (HClO) (Tan y col., 2000).Además, gracias a sus residuos O-metilo y N-acetilo, le confieren un carácter

anfipático, la molécula de melatonina es capaz de cruzar las barreras fisiológicas y por lo tanto, reducir el daño oxidativo en distintos compartimentos celulares (Bonnefont-Rousselot y Collin, 2010).

Por otro lado, también es capaz de estimular la actividad y expresión de otros sistemas antioxidantes, protegiendo del daño oxidativo por vía indirecta, a través de la activación de enzimas antioxidantes endógenos como la superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx), glutatión reductasa (GR) (Chen y col., 2013; Tamura y col., 2014). Asimismo, se ha demostrado que no sólo es la melatonina sino también los metabolitos que se forman durante estas interacciones (AFMK, AMK) son excelentes captadores de radicales libres (Galano y col., 2013).

De esta manera, su eficacia es mucho mayor que si actuara de forma independiente, como ocurre con la mayoría de antioxidantes (Reiter y col., 2000). Además es capaz de potenciar el efecto de otros antioxidantes naturales, gracias al grupo N-acetil, ya que posee acción sinérgica con la vitamina E y C (Reiter y col., 2003). Asimismo, tiene la capacidad de quelar metales, protegiendo a las células de los daños causados por los mismos (Rodríguez y col., 2004; Sofic y col., 2005; Handerland y col., 2011).

Los daños producidos por especies reactivas pueden acumularse en el tiempo e inducir daño celular, oxidando ácidos nucleicos, lípidos y proteínas (Halliwell y Gutteridge, 1999; Ilie y Margina, 2012), lo que conlleva al desarrollo de importantes alteraciones funcionales de tejidos y órganos. Esta pérdida funcional debido al estrés oxidativo suele asociarse al proceso de envejecimiento, pero también han sido implicados en el desarrollo de muchas patologías incluyendo enfermedades cardiovasculares, diabetes, algunos tipos de cáncer, y enfermedades neurodegenerativas (Duplain y col., 2008; Puddu y col., 2009; White y col., 2010; Halliwell, 2011; Lenaz, 2012; Dikalov y Nazarewicz, 2013). La incidencia de estas enfermedades crónicas representa un 31% en enfermedades cardiovasculares, un 23% en cáncer, y un 7% en enfermedades cerebrovasculares. Hasta un 60% de mortalidad es atribuible a enfermedades relacionadas con la alimentación.

Debido a la potente acción antioxidante de la melatonina, su aplicación médica es posible para la prevención o tratamiento de enfermedades relacionadas con el estrés oxidativo como son las enfermedades neurodegenerativas tales como Alzheimer (Brusco y col., 1998; Wu y Swaab, 2005), Parkinson (Tan y col., 2003), esclerosis múltiple, pérdida de memoria y depresión aunque todavía está en estudio (Coto-Montes y col., 2012). Otras enfermedades relacionadas con el estrés oxidativo son la DM, aunque los efectos farmacológicos no han sido demasiado estudiados, pero se conoce que la melatonina influye directamente en la glucosa y la secreción de insulina en las células beta (Zephy y Ahmad, 2015), produce un aumento en los niveles de

insulina durante el día, cuando la secreción de melatonina disminuye (Boden y col., 1996).

Con el objetivo de frenar este problema de salud pública y reducir los gastos económicos generados, los países deben disponer de sistemas y servicios para promover la cobertura sanitaria universal y apoyar modos de vida saludables que ayuden a prevenir y controlar la incidencia de estas enfermedades, de esta forma se puede prevenir o retrasar la aparición de enfermedades crónicas, mejorando así la calidad de vida hasta el envejecimiento.

Se han realizado gran cantidad de estudios, tanto *in vitro* como *in vivo*, sobre los efectos directos e indirectos que tiene la melatonina administrada de forma pura (Tabla 7).

Tabla 7. Administración melatonina pura en animales de experimentación.

Modelo animal	Duración	Dosis	Efecto	Referencia
SD gestantes	1,2 y 3 h	10 mg/kg peso	La administración de Mel exógena a la madre aumenta la AOX de la descendencia	(Okatani y col., 2000)
SDmacho	12 s	0,4 µg/mL en agua	Disminución peso corporal, tejido adiposo abdominal, leptina en plasma e insulina	(Wolden-Hanson y col., 2000)
SHR y WKY	6s	10 mg/mL	Reduce inflamación renal y presión arterial	(Nava y col., 2003)
SD macho	8 s	0,2µg/mL	Control peso	(Puchalski y col., 2003)
WKY macho	9 s	25µg/mL	Reducción insulina, glucosa y triglicéridos	(Ríos-Lugo y col., 2010)
WKY	10d	27,85g nutracéutico	Estabiliza lo niveles de Mel en edad avanzada	(Delgado y col., 2013)

Leyenda: h: hora, d: días, s: semanas; SD: ratas Sprague-Dawley; WKY: ratas Wistar Kyoto; SHR: ratas espontáneamente hipertensas; Mel: melatonina
Fuente: Elaboración propia.

Por todo lo anteriormente expuesto, resulta muy interesante avanzar en el conocimiento del proceso de germinación. Los alimentos germinados se están introduciendo en la dieta actual debido a su destacada composición nutricional, ya que, son fuente de proteínas, bajos en carbohidratos y ricos en vitaminas y minerales. A pesar de la gran cantidad de estudios realizados en estos alimentos, puesto que la germinación incrementa el contenido de compuestos bioactivos, apenas se conoce acerca de la biodisponibilidad de melatonina. Este trabajo aborda de forma novedosa este aspecto, es decir, no existen investigaciones anteriores que utilicen esta estrategia para incrementar el contenido de melatonina y ser una fuente dietética, así como contribuir sobre la capacidad antioxidante siendo una alternativa a la administración de preparados farmacéuticos (preparados galénicos). Esta melatonina dietética puede ser una fuente potencial natural, no sólo para incrementar los contenidos plasmáticos, sino para ejercer todas las funciones beneficiosas que es capaz de realizar sobre el organismo.

Hipótesis y Objetivos

II. HIPÓTESIS Y OBJETIVOS

El proceso de germinación permite mejorar el valor nutricional de las legumbres. La germinación incrementa el contenido de nutrientes y compuestos bioactivos con beneficios para la salud incluyendo antioxidantes de distinta naturaleza. Por tanto, los consumidores actuales, que demandan alimentos más nutritivos y saludables, han incorporado a su dieta brotes de semillas de legumbres alcanzando éstas una gran popularidad. Se requieren de más estudios para profundizar en el contenido de compuestos beneficiosos para la salud en germinados. El presente estudio representa un avance en el conocimiento de esta temática.

A la melatonina y sus metabolitos se le asocian propiedades antioxidantes entre otras propiedades beneficiosas. Existe interés en la búsqueda de fuentes dietéticas de melatonina. Una dieta rica en melatonina podría contribuir a incrementar la defensa antioxidante *in vivo* y proteger al organismo de procesos nocivos asociados al estrés oxidativo.

La germinación puede considerarse como una estrategia agronómica eficaz para incrementar el contenido en fitoquímicos beneficiosos para la salud en legumbres, en los cuales podría encontrarse la melatonina. Consecuentemente el incremento de su contenido dietético favorecerá la biodisponibilidad y contribución a la defensa antioxidante *in vivo*. Para demostrar esta hipótesis nos planteamos los siguientes objetivos principales:

1. Evaluar la eficacia del uso de la germinación como estrategia agronómica eficaz para incrementar el contenido de melatonina dietética en legumbres convencionales y hortalizas.

2. Determinar la biodisponibilidad *in vivo* de melatonina del extracto obtenido de germinados y su contribución para mejorar la defensa antioxidante plasmática.

Para alcanzar estos objetivos generales se plantearon los siguientes objetivos específicos:

1. Evaluar la influencia del proceso de germinación sobre los compuestos bioactivos más representativos y las propiedades antioxidantes presentes en cuatro especies de legumbres no convencionales para incrementar la utilidad de estas semillas.

2. Estudiar el poder antirradicálico de distintos patrones antioxidantes de referencia presentes en alimentos vegetales para comprender sus mecanismos de acción, incluyendo el compuesto melatonina.

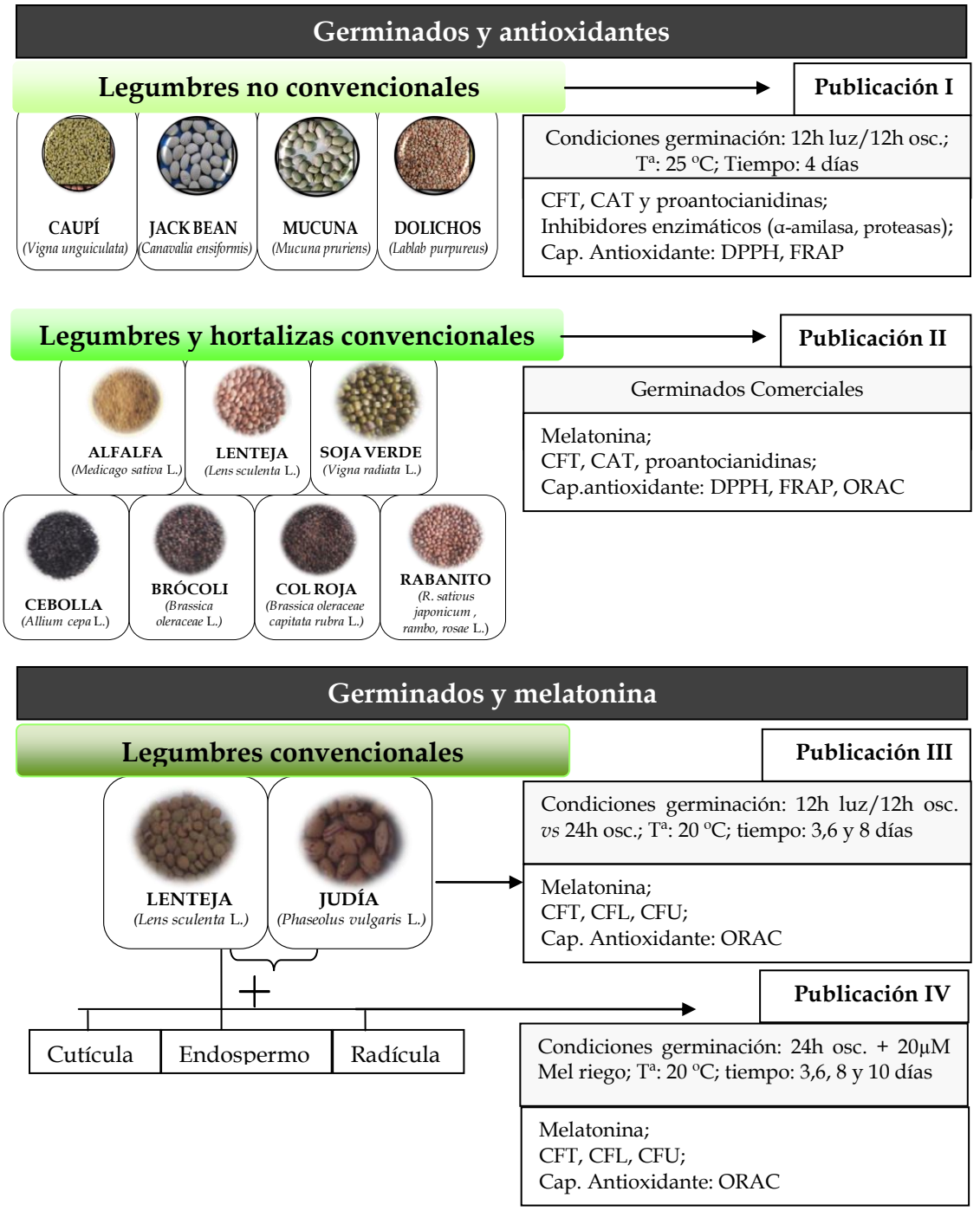
3. Determinar el contenido de melatonina en especies de semillas comestibles y sus germinados así como su capacidad antioxidante para conocer la posible contribución de la melatonina en la actividad antirradicálica de las semillas germinadas.

4. Seleccionar las mejores condiciones de germinación (12h luz/12h oscuridad o 24h oscuridad) durante 3, 6, y 8 días en dos legumbres comunes (lenteja y judía) para la consecución de germinados de leguminosas con altas concentraciones de melatonina.

5. Obtención de un extracto de germinados de leguminosas rico en melatonina y alto carácter antioxidante a partir del diseño de un proceso de germinación en el que el agua de riego se enriquece con melatonina.

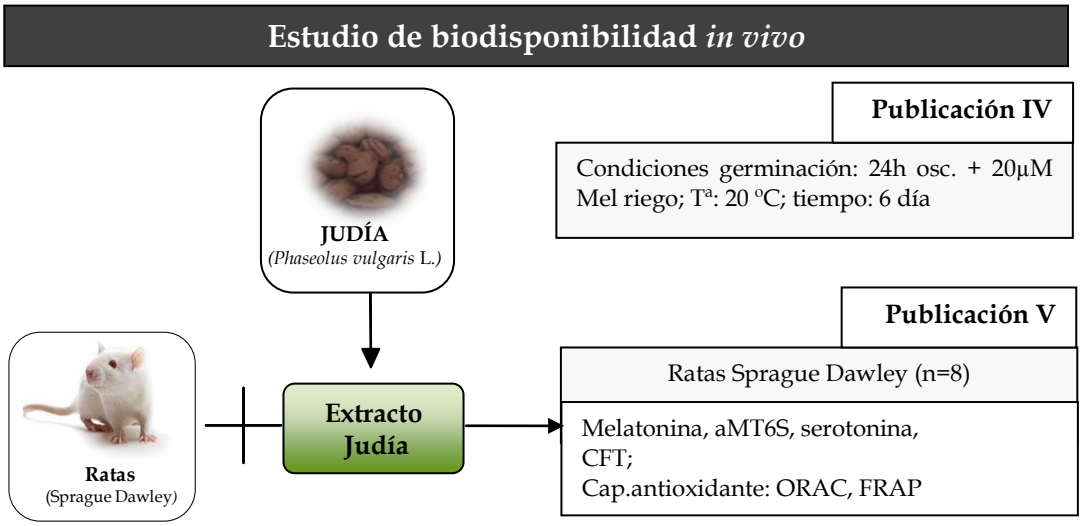
6. Evaluar la biodisponibilidad de la melatonina presente en un extracto de judía germinada mediante un ensayo *in vivo*.

Para alcanzar todos estos objetivos se planteó el plan de trabajo que se resume de manera esquemática en la figura 11.



Leyenda: h: horas; osc.: oscuridad; Mel: melatonina; CFT: compuestos fenólicos totales, CFU: compuestos fenólicos unidos, CFL: compuestos fenólicos libres, CAT: catequinas, cap.: capacidad

Figura 11. Esquema de la estrutura desarrollada en el presente estudio.



Leyenda: h: horas; osc.: oscuridad; Mel: melatonina; CFT: compuestos fenólicos totales, CFU: compuestos fenólicos unidos, CFL: compuestos fenólicos libres, CAT: catequinas, cap.: capacidad

Figura 11(cont.). Esquema de la estrutura desarrollada en el presente estudio.

Resultados

III. RESULTADOS

En esta sección se exponen los resultados más relevantes obtenidos durante el desarrollo de la presente Tesis Doctoral que se recogen en 5 artículos de investigación publicados en revistas científicas incluidas en el Science Citation Index (SCI).

Previo a la realización de la presente investigación no existía información disponible en relación al contenido de melatonina en germinados, y de su biodisponibilidad *in vivo*. La composición de la matriz alimentaria puede significativamente afectar a la biodisponibilidad de fitoquímicos alimentarios reduciendo o potenciando sus efectos. Los estudios *in vitro* deben ser completados con estudios *in vivo* para demostrar el significado biológico de los componentes alimentarios en la salud. La presente Memoria aporta información nueva en este sentido.

Para la realización de la presente Memoria se han utilizado diferentes variedades de leguminosas pertenecientes a la misma familia *Leguminosae* (Fabaceae), empleando legumbres convencionales y no convencionales. En primer lugar, se planteó como principal objetivo evaluar el comportamiento de los compuestos bioactivos más representativos presentes en legumbres germinadas, así como su contribución a la actividad antioxidante (**Publicación I**). Para ello se utilizaron legumbres no convencionales como *Vigna unguiculata* (cowpea), *Canavalia ensiformis* (jack bean), *Lablab purpureus* (dolichos), y *Stizolobium niveum* (mucuna) todas ellas de origen tropical. Tras la germinación, se incrementó la presencia de compuestos fenólicos totales, catequinas y proantocianidinas, acompañadas por un aumento general de la actividad antioxidante; por otro lado, los inhibidores de proteasas y lectinas que fueron detectados en las semillas de leguminosas, sufrieron descensos significativos tras la germinación. Por lo tanto, se observó que con el proceso de germinación se aumentaba el valor nutritivo de las legumbres estudiadas así como su capacidad antioxidante debido a una reducción de los compuestos no nutricionales y un aumento de los compuestos fenólicos, respectivamente. El alto carácter antioxidante que presentaban las leguminosas germinadas, hizo plantear si podrían estar presentes otros compuestos bioactivos antioxidantes que contribuyeran al aumento de esta actividad.

Por esta razón, en el siguiente estudio (**Publicación II)** se planteó la evaluación del poder antirradicálico de distintos patrones antioxidantes de referencia presentes en alimentos vegetales para comprender sus mecanismos de acción, incluyendo el compuesto melatonina. De esta investigación, se observó que este componente aunque posee bajo poder antirradicálico *in vitro*, su tiempo de acción es corto, por lo que podría estar implicada en los cambios metabólicos que se producen durante la

germinación. Para ello, en este trabajo se aporta nueva información relativa al contenido de melatonina en vegetales germinados de leguminosas (alfalfa, lenteja y soja verde) y hortalizas (cebolla, brócoli, col roja y rabanito) comercializadas en el mercado actualmente. La concentración de melatonina fue variable dependiendo de las distintas especies analizadas exhibiendo un incremento significativo durante la germinación. Asimismo, este proceso provocó un incremento significativo de la capacidad antioxidante, exhibiendo altas correlaciones con los compuestos fenólicos y la melatonina (r= 0,90-0,98, $p < 0,01$), contribuyendo así a la alta actividad anti-rradicálica expuesta por las semillas germinadas (hasta 97%). A la vista de los resultados expuestos en esta publicación, se marcó como próximo objetivo seleccionar las mejores condiciones lumínicas de germinación para lograr altas concentraciones de melatonina en germinados de leguminosas, lo que se traduciría en germinados de alto valor antioxidante ricos en compuestos bioactivos con posibles usos como ingredientes en la industria alimentaria.

Las condiciones de germinación tales como la iluminación tienen un gran impacto en los cambios en la composición química del alimento. Para ello se plantearon dos condiciones diferentes de iluminación durante la germinación (12h luz/12 h oscuridad vs. 24 h oscuridad) en lentejas (*Lens culinaris* L.) y judías (*Phaseolus vulgaris* L.), ya que se ha estudiado que la melatonina se sintetiza en mayores concentraciones durante la oscuridad (**Publicación III**). De este modo, el contenido de melatonina se incrementó significativamente ($p < 0,05$) tras 6 días de germinación bajo 24 h de oscuridad para ambas leguminosas. A partir de los resultados obtenidos se seleccionó para futuros estudios, un proceso de germinación realizado en su totalidad en oscuridad, ya que eran las mejores condiciones para lograr mayores contenidos de melatonina y compuestos fenólicos. La capacidad antioxidante aumentaba según avanzaba el proceso de germinación, pudiendo ser debido a la acumulación de compuestos de alto carácter antioxidante provenientes de la degradación de melatonina así como de la activación de distintas cascadas enzimáticas antioxidantes. A pesar de obtener altas concentraciones de melatonina en el sexto día de germinación, el siguiente objetivo a plantear fue aumentar en mayor proporción la concentración de este compuesto, para así obtener un extracto rico en melatonina que pudiera ser relevante desde el punto de vista fisiológico.

Para lograr dicho propósito se diseñó un proceso de germinación, donde el agua de riego se enriqueció con melatonina, estudiando sus niveles a lo largo del proceso en lentejas (*Lens culinaris* L.) y judías (*Phaseolus vulgaris* L.) (**Publicación IV**). Los primeros resultados obtenidos mostraron aumentos significativos de la concentración de melatonina, siendo más destacados entre el sexto y octavo día, y localizándose principalmente en la parte más externa de la semilla, la cubierta. Los niveles encontrados de melatonina son muy superiores a los obtenidos en la anterior

publicación, acompañados por mayores niveles de capacidad antioxidante, lo que se traduce en la obtención de un extracto con alto carácter antioxidante capaz de mejorar esta actividad en sangre. Esta hipótesis se plantea como objetivo en la última publicación abordada en esta Memoria: evaluar la biodisponibilidad de la melatonina presente en un extracto de judía germinada mediante un ensayo *in vivo* (**Publicación V**).

Para comprobar la efectividad de los germinados como fuente biodisponible de melatonina y su contribución a la defensa antioxidante se realizó un estudio *in vivo* empleando como modelo animal ratas Sprague Dawley suministrando el extracto de judía germinada obtenido anteriormente (**Publicación V**). Los resultados ponen de manifiesto que los niveles plasmáticos de melatonina se incrementaron después de la ingestión de este extracto en comparación con sus niveles basales. Este aumento está correlacionado con el contenido en orina de 6-sulfatoximelatonina, principal metabolito de degradación de melatonina de mayor capacidad antioxidante que ésta. A pesar de no encontrar aumentos significativos de la actividad antioxidante plasmática es posible que el suministro prolongado de fuentes ricas en melatonina puedan mejorar los sistemas de defensa antioxidantes en la prevención de enfermedades relacionadas con el estrés oxidativo.

Los resultados de este estudio han dado lugar a cinco publicaciones:

Publicación I. Cambios en factores no nutricionales y la actividad antioxidante durante la germinación de legumbres no convencionales

Publicación II. Estimación de la capacidad antioxidante de la melatonina y otros antioxidantes: Contribución y evaluación en semillas germinadas

Publicación III. Efecto de la iluminación en el contenido de melatonina, compuestos fenólicos y actividad antioxidante durante la germinación de lentejas (*Lens culinaris* L.) y judías pintas (*Phaseolus vulgaris* L.)

Publicación IV. Impacto del enriquecimiento de melatonina durante la germinación sobre compuestos bioactivos y actividad antioxidante

Publicación V. El consumo de germinados de judía influye sobre la melatonina y capacidad antioxidante como biomarcadores de niveles en ratas

Publicación I. Cambios en factores no nutricionales y la actividad antioxidante durante la germinación de legumbres no convencionales

Yolanda Aguilera, María Felicia Díaz, Tania Jiménez, Vanesa Benítez, Teresa Herrera, Carmen Cuadrado, Mercedes Martín-Pedrosa, and María A Martín-Cabrejas. Changes in Nonnutritional Factors and Antioxidant Activity during Germination of Nonconventional Legumes.*J. Agric. Food Chem*. **2013**, *61*, 8120−8125

RESUMEN

El presente estudio describe los efectos de la germinación sobre los factores antinutricionales y la actividad antioxidante en legumbres no convencionales *Vigna unguiculata* (cowpea), *Canavalia ensiformis* (jack bean*), Lablab purpureus* (dolichos), y *Stizolobium niveum* (mucuna). Inhibidores de proteasa y lectinas fueron detectados en las semillas de leguminosas, sufriendo descensos significativos durante el proceso de germinación. Además, se incrementó la presencia de fenoles totales, catequinas y proantocianidinas, acompañadas por un aumento general de la actividad antioxidante. Por lo tanto, la germinación ha mostrado ser un proceso muy efectivo para reducir los factores antinutricionales de estas leguminosas no convencionales, así como para aumentar la concentración de compuestos fenólicos bioactivos y su actividad antioxidante. Por esta razón, podrían ser utilizados como ingredientes para obtener harinas de leguminosas de alto valor para la formulación de alimentos.

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Changes in Nonnutritional Factors and Antioxidant Activity during Germination of Nonconventional Legumes

Yolanda Aguilera,[†] María Felicia Díaz,[‡] Tania Jiménez,[†] Vanesa Benítez,[†] Teresa Herrera,[†] Carmen Cuadrado,[§] Mercedes Martín-Pedrosa,[§] and María A Martín-Cabrejas*[,†]

[†]Instituto de Investigación de Ciencias de la Alimentación (CIAL), Facultad de Ciencias, Universidad Autónoma de Madrid, C/Nicolás Cabrera 9, Campus de Cantoblanco, 28049 Madrid, Spain

[‡]Instituto de Ciencia Animal, Carretera Central, Km 47, 1/2, San José de las Lajas, La Habana, Cuba

[§]Departamento de Tecnología de Alimentos, SGIT-INIA, Ctra. de La Coruña km 7.5, 28040 Madrid, Spain

ABSTRACT: The present study describes the effects of germination on nonnutritional factors and antioxidant activity in the nonconventional legumes *Vigna unguiculata* (cowpea), *Canavalia ensiformis* (jack bean), *Lablab purpureus* (dolichos), and *Stizolobium niveum* (mucuna). Protease inhibitors and lectins were detected in raw legumes and were significantly decreased during the germination. Regarding total and individual inositol phosphates (IP5-IP3), important reductions of IP6 and high increases in the rest of inositol phosphates were also detected during this process. In addition, total phenols, catechins, and proanthocyanidins increased, accompanied by an overall rise of antioxidant activity (79.6 μmol of Trolox/g of DW in the case of mucuna). Germination has been shown to be a very effective process to reduce nonnutritional factors and increase bioactive phenolic compounds and antioxidant activities of these nonconventional legumes. For this reason, they could be used as ingredients to obtain high-value legume flours for food formulation.

KEYWORDS: *nonconventional legumes, germination, nonnutritional factors, antioxidant capacity*

INTRODUCTION

The role of seed legumes in the diets of animals and man in developed countries is well documented.[1] There is a lack of sufficient animal protein; hence it is necessary to search for alternative sources of protein from lesser-known legumes in lieu of expensive and scarce animal protein. The research efforts are being directed to this area to identify and evaluate under-exploited legume food sources as alternative protein crops for the future. This development has stimulated research on the utilization of some underutilized legumes such as *Vigna unguiculata* (cowpea), *Canavalia ensiformis* (jack bean), *Stizolobium niveum* (mucuna), *Lablab purpureus* (dolichos), which are potential sources of plant protein for many developing countries.[2,3] The protein quality of these wild pulses seems to be similar to that of most edible legumes, and thus, they are advocated to be good sources of extending protein sources.[4] In addition, they provide a large amount of structural carbohydrates mainly due to their higher dietary fiber content when compared to other fiber rich plant foods such as cereals and tubers.[5,6] Many studies have been carried out to determine the benefits of legume dietary fiber such as prevention of obesity, cardiovascular disease, type 2 diabetes, and large intestine cancer. Thus, the role of legumes as therapeutic agents in the diet of healthy vulnerable populations (diabetes, metabolic disorders, etc.) is actually of great interest.[7]

Moreover, the uses of legumes in food formulation are assuming a greater importance and have attracted the attention of food processors, marketers, and consumers,[8] since their properties show a great impact on their utilization and are very important in the development of functional ingredients in some foods such as breads, cakes, and biscuits.[9,10]

However, their nutritional quality is limited by the presence of heat labile and heat stable nonnutritional factors (NNFs). The NNFs are structurally different compounds broadly divided into two categories: proteins (such as lectins and protease inhibitors) and others such as phytate, tannins, or proanthocyanidins, saponins, and alkaloids. The presence, distribution, and negative impact of the ingestion of NNFs in grain legumes have extensively been reported.[11] In general, raw legumes contain far higher levels of some NNFs than their processed forms, and hence processing is necessary before the incorporation of these grains into food or animal diets.[12]

In this regard, germination has been identified as an inexpensive and effective technology for improving the quality of legumes, by enhancing their digestibility increasing the content of soluble protein[13] and dietary fiber[5,6] and reducing the levels of nonnutritional factors.[14] Numerous investigations into the effects of germination on protein, starch, and dietary fiber have been carried out in common legumes. However, there is a paucity of literature about the changes of this process on underexploited legumes.[2,3] Moreover, germination conditions and their effects on legume composition can vary with the plant species, seed varieties, or cultivars.[15]

Thus, the present study was carried out with the aim to evaluate the influence of the germination process on nonnutritional factors and also antioxidant properties in four non-

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conventional legumes in order to make them more useful than the raw seeds for human and animal feed.

MATERIALS AND METHODS

Samples. Seeds of cowpea (*Vigna unguiculata* L.), jack bean (*Canavalia ensiformis* L.), dolichos (*Lablab purpureus* L.), and mucuna (*Stizolobium niveum* L.) were grown and supplied by the Instituto de Ciencia Animal (La Habana, Cuba).

Germination. The germination procedure for seeds was as follows: four portions of 150 g of seeds were washed with 0.7% sodium hypochlorite, soaked in 450 mL of distilled water at room temperature for 6 h, and shaken every 30 min. The water was then drained off, and the seeds were transferred to a separating funnel. The samples were germinated using 12 h of light daily.[4] The germination was carried out at 25 °C for 4 days, and seeds were sprayed daily with distilled water in order to maintain an adequate hydration level. The sprouts and seeds were ground and freeze-dried for analysis.

Enzyme Inhibitors. Seed flours were extracted (1:10 w/v) by stirring with 0.02 M sodium phosphate buffer pH 7.0 containing NaCl (8 g/L) overnight at +1 °C and centrifuged (5000*g* for 25 min). The resultant clear supernatants were stored at −20 °C. Estimations of protease-inhibitors content were carried out as described previously.[16] The α-amylase inhibitor content of the seed extracts was determined by the starch/iodine procedure of Piergiovanni.[17]

Lectins. Hemagglutinating activity in the pH 7.0 buffer extracts was estimated by a serial dilution procedure using rat blood cells.[18] The amount of material (g) that caused agglutination of 50% of the erythrocytes was defined as that containing 1 hemagglutinating unit (HU) and for comparison; values were expressed as HU/kg of seed meal. Trypsinized rat blood cells were used in order to detect the hemagglutination activity in legumes that showed lower activity.

Phytic Acid. The individual inositol phosphates (IP3-IP6) were extracted according Burbano et al.[19] with modifications and measured by HPLC. Analysis was with a Beckman System Gold HPLC equipped with a refractive index. The column was a macrosporous polymer PRP-1 (150 × 4.1 mm, 5 μm) heated at 45 °C and was equilibrated with the mobile phase for 1 h. The mobile phase was prepared by mixing methanol/water (51.5:48.5 v/v) with addition of 8 mL of tetrabutylammonium hydroxide (TBNOH), 1 mL of 0.5 M sulfuric acid, 0.5 mL of formic acid (ACS reagent, 91%), and 0.2 mL of a phytic acid solution (5 mg/mL). The pH was adjusted to 4.3. The mobile phase was filtered through a Millipore filter (0.45 μm) and degassed under a vacuum. The flow rate was 1.2 mL/min, and the injection volume was 20 μL. The standard used was sodium phytate (Sigma Chemicals, USA).

Polyphenolic Compounds. For extracting polyphenolic compounds, legume flours (5 g) were macerated with 3 × 50 mL of a solution of methanol-ClH ($1/_{1000}$)/water (80:20 v/v) using an orbital shaker (Stuart, Staffordshire, UK) at room temperature, separating the supernatants by centrifugation (3024*g*, 10 min, 5 °C). The three combined supernatants were taken to a fixed volume (150 mL) of the methanol solution, yielding a methanol extract in which the phenolic compound families and radical scavenging activity of the extract were determined. In the methanol solution, total polyphenols were quantificated by Folin-Ciocalteu reactant,[20] catechins with vanillin/HCl,[21] and proanthocyanidins by hydrolysis with butanol/HCl.[22]

Antioxidant Capacity. Ferric reducing antioxidant power (FRAP) assay was done according to Benzie and Strain[23] with some modifications. Legume flours (150 μL) were reacted with 2850 μL of the FRAP solution for 30 min in dark conditions. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The standard curve was linear between 25 and 800 mM Trolox. Results are expressed in μmol of Trolox/g of dry weight (DW). Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl) was done according to the method of Brand-Williams et al.[24] with some modifications. First, the absorbance of the disposable cuvette with 250 μL of the DPPH solution and 2.1 mL of 80% methanol was measured as blank. Then, the 80% methanol extracts (100 μL) were added to 250 μL of the DPPH solution and 2 mL of 80% methanol and allowed to stand at room temperature in the dark for 20 min. The decrease in absorbance of the resulting solution was monitored at 517 nm for 20 min. The Trolox standard solution (concentration 100−200 μM) in 80% methanol was prepared and assayed under the same conditions. DPPH-RSA-scavenging activity was expressed as μmol of Trolox/g of DW of sample.

Statistical Analysis. Germination was carried out in duplicate. Each sample was analyzed in triplicate. The data were analyzed by-one way analysis of variance (ANOVA) using Duncan test. Differences were considered to be significant at $P \leq 0.05$. The statistical analysis was performed by SPSS 17.0.

RESULTS AND DISCUSSION

Germination Process. The study of the effect of germination may provide useful information for optimizing of use of these legume seeds as food products, since germination has proved beneficial for the nutritional quality of common seeds. Table 1 indicates the changes in biomass and germination percentages with 12 h of light daily for 4 days. Fresh weight of seedlings increased from 113% to 340%. Cowpea showed the highest increase in fresh weight, while jack bean seeds exhibited the lowest increase. The obtained results are similar to those found for common legumes,[25] and few data were found in the literature for the nonconventional legumes.[2,5] The success of this process on these legumes was high, showing the good viability of cowpea and jack bean (98%) and dolichos (84%), while mucuna seeds only reached 63% of germination. Regarding radicle development, 96 h of the germination seemed to show high lengths in all studied legumes. Mucuna was the nonconventional legume with the smallest size of radicle (2.7 cm), while cowpea exhibited the greatest length (7.1 cm).

Table 1. Changes in Seed/Seedings Biomass and Percent of Germination at 25 °C for 4 Days

legume	% germination	development of radicle (cm)	% increase in fresh weight of seeds/seedings
cowpea	98	7.1 ± 0.3	340
jack bean	98	5.9 ± 0.2	113
dolichos	84	5.2 ± 0.5	204
mucuna	63	2.7 ± 0.2	200

Changes of Enzyme Inhibitors and Lectins during the Germination. α-Amylase and protease inhibitors are widely distributed in legumes, and their levels were influenced by the germination process (Table 2). No α-amylase inhibitor content was detected in raw legumes; only protease inhibitors that may have a major impact on nutritional value were identified. Trypsin inhibitors levels in raw seeds ranged from 1.4 mg/g of DW in mucuna to 6.9 mg/g of DW in dolichos. It was observed that colored cultivars showed lower trypsin inhibitor content, as Makkar et al.[26] established and also the different germination conditions modified the levels.[27] Regarding chymotrypsin inhibitor, the contents were lower than trypsin inhibitors, with cowpea, dolichos, and mucuna showing similar contents (1.5−1.6 mg/g of DW), higher than those exhibited by jack bean (0.7 mg/g of DW). Germination decreased significantly the trypsin inhibitor levels in all legumes except for mucuna, which did not show any change during process. Jack bean exhibited the highest reduction (78%), followed by dolichos and cowpea (54% and 38%, respectively). In the case of

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Table 2. Influence of Processing on α-Amylase and Protease Inhibitors (mg/g of DW) and Lectins Contents (mg/100 mg of DW of Lectin) in Raw and Germinated Non-Conventional Legumes[a]

samples	α-amylase inhibitor	trypsin inhibitor	chymotrypsin inhibitor	lectins[b]	lectins[c]
cowpea					
raw	n.d.	3.4 ± 0.13[b]	1.6 ± 0.08[a]	n.d.	n.d.
germinated	n.d.	2.1 ± 0.12[a]	1.5 ± 0.04[a]	n.d.	n.d.
jack bean					
raw	n.d.	3.2 ± 0.18[b]	0.7 ± 0.07[b]	5.1 ± 0.06[b]	
germinated	n.d.	0.7 ± 0.06[a]	0.4 ± 0.08[a]	2.5 ± 0.06[a]	
dolichos					
raw	n.d.	6.9 ± 0.14[b]	1.6 ± 0.10[b]	n.d.	10.2 ± 0.03[b]
germinated	n.d.	3.2 ± 0.09[a]	1.0 ± 0.10[a]	n.d.	2.5 ± 0.03[a]
mucuna					
raw	n.d.	1.4 ± 0.01[a]	1.5 ± 0.15[b]	n.d.	n.d.
germinated	n.d.	1.3 ± 0.10[a]	0.9 ± 0.08[a]	n.d.	n.d.

[a]Values are means of three analyses. n.d. not detected. Mean values within a column and legume followed by different superscript letter were significantly different at $p < 0.05$. Mean ± SD ($n = 3$). [b]Hemagglutinating unit (HU) or lectin equivalent (mg)/100 mg in legume seeds using rat blood cells. [c]Hemagglutinating unit (HU) or lectin equivalent (mg)/100 mg in legume seeds using trypsinized rat blood cells.

chymotrypsin inhibitor activity, the decreases by germination were also significant; dolichos, mucuna, and jack bean had reductions of 38%, 40%, and 43%, respectively, while cowpea did not show any noticeable decrease. Although these legumes contain significant levels of these protease inhibitors, the germination process seemed to decrease their contents. This fact might be related to different activities of endogenous proteases, due to an increase of pre-existent proteases that are activated.[14]

An initial evaluation of lectin content in all legume samples was carried out by using the hemagglutination assay. The results indicated that lectins were only detected in raw jack bean (Table 2), with levels similar to those in the literature;[16−18] in germinated jack bean a 50% decrease was observed. To detect the hemagglutination activity in the rest of legumes, trypsinized blood cells were used. Dolichos was the only legume that showed hemagglutination activity, exhibiting 76% of reduction in their germinated seeds. Hence, lectins in jack bean and dolichos were reduced by the germination treatment, improving the biological and nutritional value for their utilization in human foods and animal feeds.

Quantification of Inositol Phosphates during the Germination. Results of individual and total inositol phosphates of legume flours are shown in Table 3 and Figure 1, respectively. The total inositol phosphates contents determined by ion-pair HPLC exhibited differences among legumes ranging from 6.7 mg/g of DW (jack bean) to 8.8 mg/g of DW (dolichos). Similar contents were found in cowpea, dolichos, and mucuna (~8.4 mg/g of DW, respectively). The phytic acid composition of the studied legumes agreed with previously published data on nonconventional legumes by Sridhar and Seena[28] and Shohag et al.[29] The relative percentage values obtained of IP3-IP6 indicate that the legumes contained more than 70% of their inositol phosphates in the IP6 form, and in the case of cowpea and jack bean the value reached 83% and gave results similar to those found in the literature.[11,28,29] IP5 is the second predominant inositol phosphates in all studied legumes, ranging from 15% in jack bean to 23% in mucuna. The relative percentages of IP3 and IP4 are low and never higher than 3%. Only the highly phosphorilated inositol phosphates IP6 and IP5 have a negative effect on the bioavailability of minerals; the other hydrolytic products formed had a poor capacity to bind mineral.[30]

Table 3. Influence of Processing on Inositol Phosphates Content (mg/g of DW) of Raw and Germinated Nonconventional Legumes[a]

samples	IP3	IP4	IP5	IP6
cowpea				
raw	n.d.	0.2 ± 0.01[a]	1.3 ± 0.09[a]	6.8 ± 0.09[b]
germinated	0.1 ± 0.00	0.3 ± 0.02[b]	1.5 ± 0.06[b]	5.0 ± 0.17[a]
jack bean				
raw	0.1 ± 0.00	0.1 ± 0.01[a]	1.0 ± 0.07[a]	5.6 ± 0.12[b]
germinated	n.d.	0.6 ± 0.02[b]	1.6 ± 0.05[b]	3.5 ± 0.17[a]
dolichos				
raw	0.1 ± 0.00	0.3 ± 0.06[b]	1.9 ± 0.30[b]	6.5 ± 0.35[a]
germinated	n.d.	0.2 ± 0.03[a]	1.3 ± 0.03[a]	6.7 ± 0.25[a]
mucuna				
raw	0.1 ± 0.00	0.2 ± 0.03[a]	1.9 ± 0.11[b]	6.0 ± 0.18[b]
germinated	n.d.	0.4 ± 0.02[b]	1.6 ± 0.10[a]	5.0 ± 0.13 [a]

[a]Values are means of three analyses. Mean values within a column and legume followed by different superscript letter were significantly different at $p < 0.05$. Mean ± SD ($n = 3$).

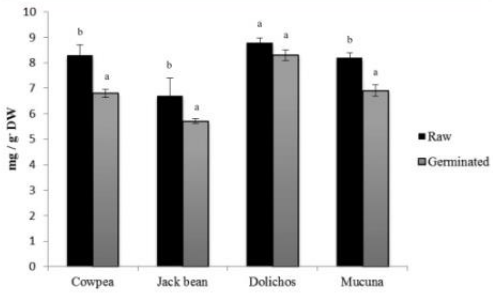

Figure 1. Influence of processing on total inositol phosphates content (mg/g of DW) of raw and germinated nonconventional legumes (mean ± SD, $n = 3$).

The germination process significantly affected the inositol phosphates contents in all seeds except for dolichos, which exhibited similar values after this treatment and the highest levels among germinated legumes. Germination caused a significant reduction ($p < 0.05$) of total inositol phosphates,

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Table 4. Influence of Processing on Total Phenols, Total Proanthocyanidins, and Total Catechins of Raw and Germinated Nonconventional Legumes[a]

samples	total phenols (mg gallic acid/g of DW)	total proanthocyanidins (mg/g of DW)	total catechins (mg/g of DW)	PC/CAT
cowpea				
raw	3.30 ± 0.11[a]	0.50 ± 0.02[a]	0.10 ± 0.01[a]	5.0
germinated	3.70 ± 0.03[b]	0.70 ± 0.02[b]	0.20 ± 0.01[b]	3.5
jack bean				
raw	2.30 ± 0.05[a]	0.02 ± 0.01[a]	0.02 ± 0.01[a]	1.0
germinated	3.60 ± 0.02[b]	0.04 ± 0.01[b]	0.07 ± 0.01[b]	0.6
dolichos				
raw	0.72 ± 0.02[a]	0.20 ± 0.02[a]	0.04 ± 0.01[a]	5.0
germinated	1.70 ± 0.03[b]	0.20 ± 0.01[a]	0.10 ± 0.01[b]	2.0
mucuna				
raw	37.40 ± 0.42[a]	0.10 ± 0.02[a]	0.03 ± 0.01[a]	3.3
germinated	46.30 ± 0.12[b]	0.20 ± 0.03[b]	0.07 ± 0.01[b]	2.8

[a]Values are means of three analyses. Mean values within a column and legume followed by different superscript letter were significantly different at $p < 0.05$. Mean ± SD ($n = 3$).

being higher in cowpea (18%), followed by mucuna and jack bean (15%). The observed reduction in phytic acid content of legume seeds during germination may be partly due to the increase of endogenous phytase activity and the corresponding hydrolysis of the IP6. In fact, this enzyme makes soluble phytates and releases soluble proteins and minerals.[31] IP6 contents decreased significantly with a reduction of 17% to 37% of the original value; no change in IP6 was noted for dolichos, but there was an increase in the other inositol phosphates (IP4-IP5). Therefore, germinated nonconventional legumes seemed to present a better potential for mineral availability when compared to raw legumes; however, the extent of reduction of inositol phosphates is dependent on the type of legume.[32] A direct correlation between phytate reduction and phytase activity was found during germination of lentils; however, in chickpeas no correlation was established.[31]

Phenolics Contents during the Germination Process. Table 4 shows the influence of the germination process on total phenolics compounds (TPC), total proanthocyanidins (PC), and total catechins (CAT). Mucuna showed the highest amounts of TPC (37.40 mg/g of DW), followed by cowpea and jack bean, while dolichos exhibited the least total phenolic content (0.72 mg/g of DW). These results may be the cause of the different color of seed coats exhibited by these legumes. Similar behavior was observed in dark and highly pigmented bean varieties reported by Xu and Chang.[33] As was observed for other bioactive compounds, the influence of processing is relevant to total phenolic contents. Germination brought about further significant increases in TPC contents of all nonconventional legumes; increases varied from 12% (cowpea) to 136% (dolichos). These results are in agreement with those reported by earlier authors[13,34−36] who found relevant increases of total phenols in germinated legumes. In contrast, Khandelwal et al.[37] detected reductions in the concentration of total polyphenols in Indian pulses. During germination, endogenous enzymes are activated, promoting differences in the composition of legume varieties in terms of phenolic compounds.

Total proanthocyanidins have also been identified in raw legumes, representing from 28% to 19% of total phenols in dolichos, mucuna, and cowpea, respectively. After germination, PC increased significantly in mucuna (50%) and cowpea (40%); however, similar levels were found in the case of dolichos. During the germination process, proanthocyanidins could condense to highly polymerized forms through the activation of polyphenoloxidase.[32] The presence of catechins in raw legumes is low compared to common legumes as the levels represented <5% of total phenols in studied seeds (Table 4). As a consequence of germination, the CAT contents increased in all pulses. This could be attributed to the production of some secondary plant metabolites,[34,38] such as flavonoids. The ratio proanthocyanidin/catechin, a relative approximation of the polymerization degree of proanthocyanidins, showed decreases in all germinated samples (Table 4). It is important from a nutritional point of view that the degree of polymerization of proanthocyanidins should be low because the proanthocyanidin−protein interactions increases with the degree of polymerization.[39] The decrease in this process could be related to several factors such as changes in their real solubility and chemical reactivity that may modify the effectiveness of the analysis.[36] A decrease in the high polymerized proanthocyanidins was also reported for the germination and fermentation of lentils.[38]

Total Antioxidant Capacity during the Germination. The antioxidant capacity of ungerminated seeds, measured by FRAP assay (Figure 2a), ranged from 1.8 to 55.3 μmol of Trolox/g of DW, while the antioxidant capacity against DPPH-free radical (Figure 2b) ranged from 1.4 to 8.6 μmol of Trolox/g of DW (Table 4). In both assays, the highest antioxidant capacity corresponded to mucuna legume and the lowest to jack bean and dolichos. The initial antioxidant capacity in raw seeds is similar to common legumes reported by Vernaza et al.[13] and Xu and Chang.[33] An enhancement in antioxidant capacity by germination was exhibited in all pulses except in jack bean, and the increases were significant ($p < 0.05$) from 89% in cowpea to 22% in dolichos. The highest antioxidant activity was seen in germinated mucuna by both methods (79.6 μmol of Trolox/g of DW by FRAP and 10.70 μmol of Trolox/g of DW by DPPH), followed by cowpea and dolichos. A similar tendency after the germination process has been reported in the literature.[13,36] Mixtures of phenolics can exhibit an enhanced antioxidant activity since they can work together synergistically, and also the germination process might produce some secondary plant metabolites such as anthocyanins and flavonoids from seed coats and cotyledons due to the enzymatic reaction.[38] Germination has been reported to lead to the production of bioactive compounds with potent antioxidant properties.[15]

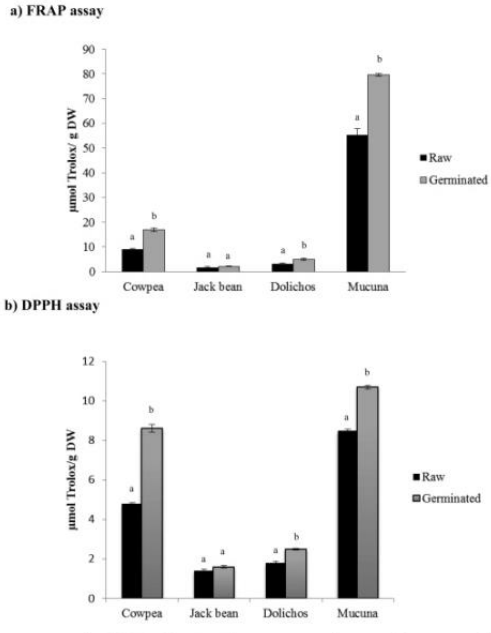

Figure 2. (a, b) Total antioxidant activity of raw and germinated nonconventional legumes (mean ± SD, $n = 3$).

Correlation analyses between total phenolic contents and antioxidant activities (FRAP and DPPH assays) were performed; these analyses exhibited significant linear correlations ($r = 0.98$, $P < 0.05$ and $r = 0.97$, $P < 0.05$, respectively). High correlation between these compounds and their antioxidant activity was also found in conventional legumes.[13,36] Moreover, significant linear correlation between DPPH and FRAP methods ($r = 0.86$, $P < 0.01$) was found with a combination of ungerminated and germinated legumes. Overall germinated legumes exhibited a more potent ability to inhibit reactions promoted by oxygen or peroxides than the raw seeds and showed a good correlation between antioxidant assays and total phenols content.

In conclusion, germination is a simple technological process that is easy to apply and has minimal cost. This process reduces enzyme inhibitors, lectins, and inositol phosphates, thereby improving the nutritional quality. In addition, a significant increase of phenolic compounds accompanied by an overall increase of antioxidant activity was observed in all germinated legumes. The germination process may be considered as an effective and promising method increasing the bioactivity of these nonconventional legumes. Nevertheless, further work to control the conditions of germination to obtain an optimal concentration of nonnutritional factors and antioxidant activity is needed to highlight the potential value of these nonconventional legumes as ingredients.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34 91 0017913. Fax: +34 91 0017905. E-mail: maria.martin@uam.es.

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Publicación II. Estimación de la capacidad atrapadora de radicales de la melatonina y otros antioxidantes: contribución y evaluación en semillas germinadas

Yolanda Aguilera, Teresa Herrera, Vanesa Benítez, Silvia M. Arribas, Ángel L. López de Pablo, Rosa M. Esteban, María A. Martín-Cabrejas. Estimation of scavenging capacity of melatonin and other antioxidants: Contribution and evaluation in germinated seeds. *Food Chemistry*. **2015**, *170*, 203–211

RESUMEN

En siete semillas germinadas comerciales se evaluaron los niveles de melatonina, compuestos fenólicos y su capacidad antioxidante. Asimismo, se midió el poder antirradicálico de patrones antioxidantes para comprender sus mecanismos de acción. La germinación provocó un incremento significativo sobre el total de los compuestos fenólicos en todas las semillas estudiadas, mostrando la col roja, rabanito y brócoli los mayores contenidos (21,6; 20,4 y 16,4 mg GAE/g m.s., respectivamente). La concentración de melatonina es muy variable en las semillas estudiadas, exhibiendo un incremento significativo durante la germinación. Los niveles más altos fueron encontrados en col roja (857 pg/g m.s.), rabanito (536 pg/g m.s.) y brócoli (439 pg/g m.s.). Las semillas germinadas con elevados niveles de polifenoles y melatonina mostraron una relevante actividad antirradicálica (>97%). Esta información resulta ser útil para la incorporación semillas germinadas de col roja, rabanito y brócoli en la dieta y así, promover potenciales beneficios para la salud.

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Estimation of scavenging capacity of melatonin and other antioxidants: Contribution and evaluation in germinated seeds

Yolanda Aguilera [a], Teresa Herrera [a], Vanesa Benítez [a], Silvia M. Arribas [b], Angel L. López de Pablo [b], Rosa M. Esteban [a], María A. Martín-Cabrejas [a,*]

[a] Instituto de Investigación de Ciencias de la Alimentación (CIAL), Facultad de Ciencias, Universidad Autónoma de Madrid, C/Nicolás Cabrera 9, 28049 Madrid, Spain
[b] Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, 28049 Madrid, Spain

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ABSTRACT

Seven edible seeds for the levels of melatonin, phenolic compounds and their antioxidant capacity were evaluated during germination process. Radical scavenging parameters were also studied in standard antioxidants to understand their antiradical actions. Germination brought about significant increases of total phenol compounds in all edible seeds, showing red cabbage, radish and broccoli the highest contents (21.6, 20.4 and 16.4 mg GAE/g DW, respectively). The concentration of melatonin is greatly variable in edible seeds, exhibiting significant increases during germination. The highest levels were found in red cabbage (857 pg/g DW) radish (536 pg/g DW) and broccoli (439 pg/g DW). The germinated seeds which had the highest levels of polyphenols and melatonin were those that showed the most relevant antiradical activities (>97%). This information is valuable for the incorporation of red cabbage, radish and broccoli germinated seeds into the diet to promote potential health benefits.

1. Introduction

Free radicals and other reactive oxygen species (ROS) are responsible for damages in almost all biomolecules, and participate in the development of many disorders such as atherogenesis, neurodegeneration, cardiovascular diseases, cancer as well as physiological senescence. Antioxidant compounds have specific pathways to overcome these adverse effects, showing health benefits since they neutralise the free radical excess and maintain cellular homeostasis (Slavin & Lloyd, 2012). Several types of antioxidant molecules have been found in plants; many of these are considered as phytochemicals because of their beneficial health effects. Phenolic compounds have been widely studied, due to their protection against diseases that may be associated with the powerful antioxidant and free radical scavenging properties of these compounds (Del Rio et al., 2013). However, it has been established other antiradical compounds with significant antioxidant properties, like melatonin (N-acetyl-5-methoxytryptamine), and other indoleamine compounds, which occur ubiquitously in nature and they may work in a coordinated manner on total antioxidant capacity (Allegra, Reiter, & Tan, 2003). The antioxidant properties of melatonin and its ability to scavenge several radical species as part of its bioactivity have been described in the literature (Reiter et al., 2003). Melatonin has been found to neutralise the most toxic oxidising agents generated within cells, such hydroxyl radical ($^{\cdot}OH$) and peroxynitrite anion ($ONOO^-$). In addition, melatonin reportedly scavenges singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\cdot}$) and hypochlorous acid (HClO). Several metabolites formed when melatonin interacts with radicals are also highly effective scavengers (Milczarek, Hallmann, Sokolowska, Kaletha, & Klimek, 2010). These products include cyclic 3-hydroxymelatonin, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK). Melatonin can cross physiological barriers and reduces the oxidative damage in both lipid and aqueous cell environments. Besides its ability to direct scavenge radicals and radical products, melatonin also stimulates the activity of protective antioxidant enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione reductase (Reiter, Tan, & Fuentes-Broto, 2010). Melatonin participates in the regulation of biological rhythms, vascular tone, and has oncostatic and immunomodulatory actions and hormonal regulation (Miller, Pandi-Perumal, Esquifrino, Cardinali, & Maestroni, 2006). Melatonin has been detected in plant foods such as vegetables, fruits, cereals and in some legumes (Korkmaz, Reiter, Tan, & Manchester, 2011). Nowadays, it has been also studied in some processed foods such as olive oil, wine, beer and juices

* Corresponding author. Tel.: +34 91 497 8678; fax: +34 91 497 3826.
E-mail address: maria.martin@uam.es (M.A. Martín-Cabrejas).

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(Maldonado, Moreno, & Calvo, 2009; Rodríguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011). Therefore, its incorporation into the organism (and its related indolic compounds) may improve human and animal health, by virtue of their biological activities (Reiter et al., 2010).

Considering melatonin levels in food are low, it may be interesting to find food processing technologies that not only reduce initial levels of this neurohormone but also increase them. In this sense, germination process generally improves the nutritional quality of seeds, not only by decreasing the antinutrient components (Vernaza, Dia, González De Mejía, & Chang, 2012) but also by increasing the levels of free amino acids, dietary fibre as well as the seed functionality by the subsequent increases in bioactive compounds and antioxidant properties (Allegra et al., 2003). Germinated seeds are becoming a popular health food recommended for human diet due to their advantages and consumers are demanding fresh and safe vegetables that may promote health and well-being.

Therefore, the aim of this study is to evaluate the content of melatonin and polyphenols in 7 different edible seeds during germination process in order to select suitable seeds to provide the maximum concentration of health-promoting compounds. Further, their antioxidant activity was determined by three different tests [FRAP (ferric-reducing/antioxidant power), DPPH (1, 1-diphenyl-2-picrylhydrazyl), and ORAC assay (oxygen radical absorbing capacity)] for the proper evaluation of radical scavenging activity in raw and germinated seeds. In this sense, radical scavenging parameters were also studied in several standard antioxidants present in seeds to provide us a better understanding of antiradical actions of antioxidants.

2. Materials and methods

2.1. Chemicals and materials

Gallic, chlorogenic, ferulic, *p*-coumaric acids, (+)-catechin, kaempferol, quercitin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 1,1-diphenyl-2-picrylhydrazyl (DPPH), were purchased from Sigma–Aldrich, St. Louis, USA; ascorbic acid and 2,4,6-tripyridyl-*s*-triazine (TPTZ) from Fluka Chemie (Buchs, Switzerland). All reagents were of analytical grade.

Raw and germinated seeds were supplied by the industry Aunatura Land S.A. (Madrid, Spain). Three varieties of legumes: alfalfa (*Medicago sativa* L.), lentil (*Lens sculenta* L.) and mung bean (*Vigna radiata* L.), and four varieties of vegetables: onion (*Allium cepa* L.), broccoli (*Brassica oleraceae* L.), red cabbage (*Brassica oleraceae capitata rubra* L.) and mixing of radish (*Raphanus sativus japonicum* L., *Raphanus sativus rambo* L., *Raphanus sativus sinicum rosae* L.) were studied. Samples were freeze-dried and were milled to flour and passed through a 50 μm sieve.

2.2. Kinetic measurements

The kinetic reaction was determined following the method of Brand-Williams, Cuvelier, and Berset (1995) in antioxidant standards and plant material samples. The decrease in absorbance was determined at 517 nm at 0 min and every min until the reaction reached a steady state. The decrease as a function of time is exponential in nature and plotted for different antioxidant concentrations. Additionally, the samples were determined like antioxidant solutions and the changes in DPPH· solutions were monitored over time.

The percentage of DPPH· remaining in the steady state was determined as

$$\%\ \text{DPPH remaining} = \frac{A_f}{A_o} \times 100$$

where A_0 and A_f are the radical absorbances at 517 nm at the beginning and at steady state, respectively. The reaction was measured using methanol as blank.

2.3. Estimation of antiradical properties

The antiradical properties were determined in antioxidant standards. The effective concentration (EC_{50}) value, defined as the efficient concentration required to decrease the initial DPPH· concentration by 50%, was evaluated. The concentrations of antioxidants in the reaction medium were plotted against the percentage of the remaining DPPH at the end of the reaction in order to obtain the EC_{50}. This value was expressed in terms of molar ratio of antioxidant to DPPH·, the shorter the EC_{50} the more efficient the antioxidant. Antioxidant reducing power (ARP) was the inverse of EC_{50} value, the larger the ARP the more efficient the antioxidant. Stoichiometry (number of DPPH· mole required to reduce one antioxidant mol) was calculated from EC_{50} values. The number of reduced DPPH· was the inverse of stoichiometry. T_{EC50} is the time taken to reach steady state at the concentration corresponding to EC_{50}. It was obtained by plotting the times taken to reach the steady state against the concentration of the antioxidant compound. Antiradical efficiency (AE) combines EC_{50} and T_{EC50} factors, and was calculated as:

$$\text{AE} = (\text{TEC}_{50} \times \text{EC}_{50})^{-1}$$

2.4. Polyphenolic compounds

Plant material (raw and germinated seed flours) were macerated with 7.5 mL of a solution of methanol-HCl (1‰) in orbital shaker at room temperature, the homogenates were centrifuged at 4200 rpm for 15 min, and the supernatants were filtered and stored at −20 °C until further analysis. Extractions were performed in 3 replicates for each individual sample-solvent combination. In the methanol solution, the total polyphenol content (TPC) was determined by a Folin–Ciocalteu assay according to Singleton, Orthofer, and Lamuela-Raventós (1999) using gallic acid (GA) as standard. Catechins were quantified with vanillin/HCl (Swain & Hills, 1959) and proanthocyanidins through hydrolysis with butanol/HCl (Ribereau-Gayon & Stonestreet, 1965).

2.5. Melatonin extraction and measurement by ELISA

Extraction procedure was conducted according to Manchester et al. (2000) with some modifications. Briefly, raw and germinated seed flours (2 g) were extracted with 10 mL of methanol for 16 h at 4 °C in darkness. After a brief centrifugation at 4200 rpm for 15 min at room temperature; the supernatants were filtered under vacuum by a filter (11 μm, Whatman) and they were evaporated to dryness under the nitrogen gas. The residues were redissolved in 2 mL of distilled water, and ultrasonic treatment for 2 min for further extraction of melatonin using the solid phase extraction (SPE, cartridge C-18, Waters). The extracts obtained were evaporated to dryness by use of evaporator centrifuge (Speed Vac SC 200, Savant, USA). The residues were dissolved in distilled water and melatonin levels were determined by a competitive enzyme immunoassay kit (Melatonin ELISA, IBL-International, Hamburg, Germany) according to the manufacturer's instructions. The kit is characterised by an analytical sensitivity of 1.6 pg/mL and high analytical specificity (low cross-reactivity).

2.6. Antioxidant capacity assessment

Three methods, ORAC, FRAP and DPPH, were used to evaluate the antioxidant capacity in the above methanol-HCl extracts.

2.6.1. ORAC assay: oxygen radical absorbing capacity assay

The methanol extracts of the samples were used for determining the radical scavenging activity by the ORAC method using fluorescein as a fluorescence probe (Dávalos, Gómez-Cordoves, & Bartolomé, 2004). Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained fluorescein (70 nM), 2, 2-azobis (2-methyl-propionamidine)-dihydrochloride (12 mM), and antioxidant (Trolox [from 10 to 80 μM] or sample [at different concentrations]). A Polarstar Galaxy plate reader (BMG Lab technologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy software version (4.31-0) for fluorescence measurement. Black 96-well untreated microplates (PS Balck, Porvair, Leatherhead, UK) were used. The plate was automatically shaken before the preincubated, and the fluorescence was recorded every minute for 80 min. All reaction mixtures were prepared in duplicate and at least 3 independent runs were performed for each sample. Fluorescence measurements were normalised to the oxidation control (phosphate buffer) and stability control (no antioxidant). From the normalised curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \text{AUC antioxidant} - \text{AUC blank}$$

The net AUC was plotted against the antioxidant concentration and the regression equation of the curve was calculated. The ORAC value was obtained by dividing the slope of the latter curve between the slopes of the Trolox curve obtained in the same assay. Final ORAC values were expressed as μmol of Trolox equivalents/g of dry sample (μmol TE/g DW).

2.6.2. FRAP assay: ferric reducing antioxidant power assay

The FRAP assay was performed as previously described (Benzie & Strain, 1996). Three millilitres of a working FRAP reagent [acetate buffer 0.3 M pH 3.6, 10 mM tripyridyl s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ (10:1:1) (v/v/v)] were warmed to 37 °C, and then 100 μL of methanol extracts were added. The absorbance was taken at 593 nm against reagent blank after 10 min. FRAP values were calculated and expressed as micromole of Trolox equivalent per gram of dry matter (μmol TE/g DW).

2.6.3. DPPH assay: free radical scavenging activity

DPPH free radical scavenging capacities of standards and plant material extracts were evaluated according to Xu and Chang (2007). Fifty microlitres of different standards concentrations (ranging from 50 to 2000 μM) and different raw or processed sample extracts were added to 1950 μL methanol solution of DPPH radical (6×10^{-5} mol/L). The mixture was shaken vigorously by vortexing and the decrease in absorbance (517 nm) was monitoring until to obtain a steady state and also reaction kinetics of different standards and samples. DPPH scavenging activity was expressed as μmol Trolox/g DW of sample.

2.7. Statistical analysis

Each sample was analysed in triplicate. The data were analysed by-one way analysis of variance (ANOVA) using Duncan test. Differences were considered to be significant at $p \leqslant 0.05$. The statistical analysis was performed by SPSS 17.0. Additionally, for curve-fitting analysis in ELISA assay, dates were processed by 4-Parameter Logistic nonlinear regression model.

3. Results and discussion

Before studying melatonin and polyphenol contents in raw and germinated seeds, it is necessary to evaluate the kinetic behaviour and radical scavenging parameters of selected antioxidants present in plant material in order to provide more information about antioxidant activities.

3.1. Kinetic behaviour of antioxidants

The kinetics of scavenging of DPPH free radical by the main antioxidants present in seeds, such as polyphenols (gallic acid, chlorogenic acid, ferulic acid, p-coumaric acid, catechin, kaempferol and quercitin) and indole compound (melatonin) as well as standard antioxidants (ascorbic acid and Trolox) were investigated for comparing the scavenging activity. The antioxidant concentrations (Trolox and melatonin) were plotted against the percentages of the remaining DPPH· at the end of the reaction, and the kinetic assays were performed for individual antioxidants till steady state saturation (maximum decrease in DPPH· radical attained) (Fig. 1). The time course of scavenging of DPPH free radical by Trolox in methanol (150–800 μM) is showed in Fig. 1a. The reaction shows a fast decay in absorbance in the first 5 min, followed by a slower step in which degradation products are involved until the reaction was completed at 20 min. This compound is the main standard antioxidant studied to estimate antiradical properties in food due to its high water soluble activity (Mishra & Ojha-Chaudhury, 2012). Melatonin showed a steady state reaction followed up to 10 min and relevant differences with concentrations (300–2000 μM), then reacted quickly with DPPH· (Fig. 1b). In both cases, Trolox and melatonin showed similar behaviour, the higher concentrations of antioxidant, the greater percentages of inhibition of DPPH· remanent decreases. However, these percentages reveal that Trolox shows higher antiradical activity than melatonin at the same concentration; 300 μM of Trolox reached 40% of DPPH· inhibition while 300 μM of melatonin only achieved 5%. This different behaviour could be due their different functional groups to scavenge reactions (Rodríguez-Naranjo, Moya, Cantós-Villar, & García-Parrilla, 2012; Sharma & Bhat, 2009). Thus, the indoleamines are not good peroxyl radical-trapping antioxidants *in vitro* due to the absence of hydroxyl groups, but it has been reported melatonin ability to scavenge peroxyl radical *in vivo* (Reiter et al., 2003). Antioxidant activity of melatonin, unlike other antioxidant compounds, is not only based on its ability to scavenge radicals species, but also is able to increase the activities of antioxidative enzymes and the degradation products are highly effective scavengers (AFMK, AMK and cyclic 3-hydroxymelatonin) (Korkmaz et al., 2011; Reiter et al., 2010).

To evaluate scavenging of DPPH radical by indolamine, polyphenols and common antioxidants used as standards, the kinetic reactions at 300 μM (Fig. 1c) were represented. The antioxidants showed intermediate kinetics, standing out the fast reaction of ascorbic acid with DPPH· to attain complete response within 5 min, in a similar inhibition percentage than Trolox. Ascorbic acid was, essentially, instantaneous as reported (Sharma & Bhat, 2009). The reaction of DPPH· with quercetin and gallic acid was also quite fast but slower as compared to ascorbic acid. The results are in agreement to those found by Anissi, El Hassouni, Ouardaoui, and Sendide (2014). These polyphenols needed 60 min to reach the steady state although their kinetics exhibited the highest antiradical activities (70% of DPPH· inhibition). On the other hand, p-coumaric and melatonin showed the lowest antiradical activity (less than 10%). These results suggest the difference on time

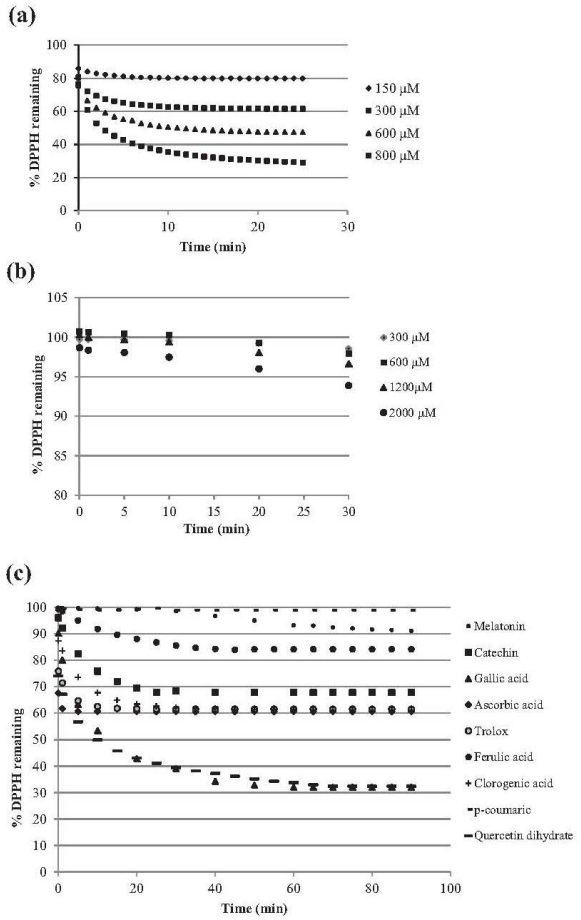

Fig. 1. (a) Time course of scavenging of DPPH free radical by Trolox (150–800 μM); (b) melatonin (300–2000 μM); (c) different antioxidants at 300 μM.

duration of reaction to reach the steady state depends on the chemical nature of antioxidant.

In addition, EC_{50} was determined at different time intervals (5, 10, 20 and 40 min). The lower EC_{50}, the higher the antioxidant activity of a compound is. When the EC_{50} remained the same after consecutive reaction time intervals, the antioxidant attains completion of reaction. As can be seen in Table 1, EC_{50} values are extremely diverse, being gallic acid, quercitin and ascorbic acid the most potent scavenging compounds and melatonin, p-coumaric acid and kaempferol, the less. Ascorbic acid is the compound that requires the minor time scavenging DPPH· radical (5 min); melatonin, catechin, kaempferol and Trolox attain completion of reaction within 10–25 min; finally the rest of antioxidants, gallic, chlorogenic, ferulic, p-coumaric, and quercitin reach a steady state within 55–65 min. Our data were similar to earlier reports (Rodríguez-Naranjo et al., 2011). Therefore, powerful antioxidants are not always acting fast while less potent compounds might have the advantage to work quickly and more efficiently in some biological reactions.

3.2. Radical scavenging parameters

The radical scavenging parameters of melatonin, polyphenols and standard antioxidants are shown in Table 2. Antioxidant reducing power (ARP) was the inverse of EC_{50} value, the larger the ARP the more efficient the antioxidant is. The highest ARP values were exhibited by gallic acid and quercitin (15.8 and 13.6, respectively), whereas the corresponding EC_{50} values were 0.06 and 0.07. Chlorogenic acid, ferulic acid, catechin, ascorbic acid and Trolox showed lower ARP values (from 4.3 to 7.4) and EC_{50} (from 0.1 to 0.2), similar to those reported by earlier publications (Sharma & Bhat, 2009). ARP values for melatonin, p-coumaric acid and kaempferol were the lowest from 0.01 to 0.07 and thus, the highest EC_{50} values from 13.8 to 85.5. The profile of polyphenols is positively correlated with the number of hydroxyl groups; a correlation is exhibited by melatonin due to the low DPPH scavenging capacity and the absence of hydroxyl groups; this observation explains our data, except for kaempferol. Trolox shows only one available hydroxyl group to react with free radicals; thus, its high

Table 1
Comparative EC_{50} (μM) values of different antioxidants at specific time points.

Compound	Time (min)			
	5	10	20	40
Indole				
Melatonin	1164.1 ± 311.8[a]	829.8 ± 108.3[a]	NC	NC
Polyphenols				
Phenolic acids				
Benzoics acids				
Gallic acid	4.7 ± 0.6[a]	4.2 ± 0.4[a]	3.5 ± 0.4[a]	3.8 ± 0.0[a]
Cinnamics acids				
Chlorogenic acid	17.6 ± 2.2[b]	12.5 ± 1.2[a]	9.7 ± 0.7[a]	11.2 ± 1.5[a]
Ferulic acid	28.9 ± 2.5[a]	23.7 ± 3.0[a]	19.3 ± 1.8[a]	16.9 ± 2.4[a]
p-coumaric acid	2444.9 ± 32.5[d]	2323.9 ± 5.8[c]	1601.2 ± 0.3[b]	1555.8 ± 0.7[a]
Flavan-3-ols				
(+)-Catechin	24.6 ± 1.4[c]	16.6 ± 0.8[b]	12.1 ± 0.0[a]	NC
Flavonols				
Kaempferol	11116.8 ± 673.5[b]	5149.1 ± 326.7 [a]	NC	NC
Quercitin	5.2 ± 1.2[a]	4.8 ± 0.9 [a]	4.5 ± 0.5[a]	4.3 ± 0.0[a]
Standard antioxidants				
Ascorbic acid	8.0 ± 0.1[a]	NC	NC	NC
Trolox	10.6 ± 0.4[c]	9.6 ± 0.2[b]	8.9 ± 0.3[a]	NC

Mean values within a row and antioxidant compounds followed by different superscript letter were significantly different at $p < 0.05$. Means ± SD ($n = 3$). NC = not changed.

Table 2
Radical scavenging parameters (EC_{50}, ARP, T_{EC50} and AE), stoichiometry and No of reduced DPPH in melatonin, polyphenols and standard antioxidants.

Compound	EC_{50} (μmol antioxidant/ μmol DPPH)	ARP	Stoichiometry	N_o of reduced DPPH	T_{EC50}	μmol Trolox/ μmol antioxidant	–OH groups
Indole							
Melatonin	13.83 ± 1.80[d]	0.07 ± 0.01[c]	27.66 ± 3.61[e]	0.02 ± 0.02[b]	10	0.01 ± 0.01[a]	0
Polyphenols							
Phenolic acids							
Benzoics acids							
Gallic acid	0.06 ± 0.00[a]	15.86 ± 1.30[h]	0.13 ± 0.01[a]	7.93 ± 0.65[e]	60	2.50 ± 0.24[c]	3
Cinnamics acids							
Chlorogenic acid	0.14 ± 0.01[b]	7.21 ± 0.58[a]	0.28 ± 0.02[c]	3.61 ± 0.69[d]	55	1.10 ± 0.07[b]	4
Ferulic acid	0.23 ± 0.02[c]	4.32 ± 0.31[d]	0.47 ± 0.04[d]	2.16 ± 0.21[c]	65	0.65 ± 0.05[a]	1
p-coumaric acid	26.69 ± 0.00[e]	0.04 ± 0.00[b]	53.38 ± 0.01[f]	0.02 ± 0.00[b]	20	N.D.	1
Flavan-3-ols							
(+)-Catechin	0.14 ± 0.01[b]	7.28 ± 0.28[f]	0.27 ± 0.01[c]	3.64 ± 0.14[d]	25	1.10 ± 0.04[b]	5
Flavonols							
Kaempferol	85.81 ± 5.63[f]	0.01 ± 0.00[a]	171.64 ± 11.5[g]	0.006 ± 0.0[a]	10	N.D.	4
Quercitin	0.07 ± 0.01[a]	13.62 ± 0.93[g]	0.15 ± 0.01[b]	6.81 ± 0.47[e]	60	2.14 ± 0.09[a]	5
Standard antioxidants							
Ascorbic acid	0.13 ± 0.00[b]	7.41 ± 0.12[f]	0.27 ± 0.00[c]	3.71 ± 0.06[d]	5	1.15 ± 0.05[b]	2
Trolox	0.15 ± 0.01[b]	6.68 ± 0.23[e]	0.30 ± 0.01[c]	3.34 ± 0.11[d]	20	1	1

EC_{50} (the antioxidant concentration that caused 50% decreased in DPPH. estimated at steady state, μmol antioxidant/μmol DPPH), ARP (antiradical power $1/EC_{50}$), Stoichiometry ($EC_{50} \times 2$), No of reduced DPPH (1/stoichiometry), T_{EC50} (time taken to reach steady state at the concentration corresponding to EC_{50}), AE (antiradical efficiency $1/T_{EC50} \times EC_{50}$), number of –OH groups available.
Mean values within a column and antioxidant compound followed by different superscript letter were significantly different at $p < 0.05$. Means ± SD ($n = 3$). N.D. no detected.

activity involves a more complex action mechanism (Friaa & Brault, 2006).

The stoichiometry, obtained by multiplying the EC_{50} by 2, determines the amount of antioxidant required to reduce 100% of DPPH· radicals (Table 2). Apart from that, these values are also presented as their inverse values (No of reduced DPPH·). On the basis of the results obtained, the order of stoichiometry values was gallic acid < quercitin < catechin = ascorbic acid < chlorogenic acid < Trolox < ferulic acid < melatonin < p-coumaric acid < kaempferol. It is worth pointing out, 1 mol of gallic acid reduces 7.93 mol of DPPH, in contrast to melatonin and p-coumaric acid, which can only reduce 0.02 mol of DPPH.

In addition, T_{EC50}, time at equilibrium reached with a concentration of antioxidant equal to EC_{50}, could help to classify the kinetic behaviour of the antioxidant compounds as follows: rapid ($T_{EC50} < 5$ min), intermediate ($5 \leqslant T_{EC50} \leqslant 30$ min) and slow ($T_{EC50} > 30$ min) (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998). According to this classification, ascorbic acid is a rapid antioxidant; melatonin and kaempferol are considered intermediates ($T_{EC50} = 10$ min) probably because complex mechanisms are involved (Table 2). This characteristic is important in biological systems, since free radicals have very short half-lives (Reiter et al., 2003). Melatonin acts quickly and may be sufficient to exert its antioxidant potential before being metabolised. Further, the kinetic reactions of p-coumaric, Trolox and catechin ($T_{EC50} = 20$–25 min) correspond to intermediate antioxidants, being faster than the rest of the polyphenols (gallic, chlorogenic, ferulic and quercetin) that can be considered slow as reported in literature (Rodríguez-Naranjo et al., 2012).

EC_{50} values as well as the time of reactivity of antioxidant (T_{EC50}) are key parameters to evaluate antiradical efficiency (AE). The highest AE value corresponds to ascorbic acid (1.54), being

Table 3
Content of phenolic compounds, catechins, proanthocyanidins and melatonin in raw seeds and germinated seeds.

Common name	Scientific name	Sample	Total phenolic compounds (mg GAE/g DW)	Total catechins (mg CAE/g DW)	Total proanthocyanidins (mg Cl Cyanidin/g DW)	Melatonin (pg/g DW)
Alfalfa	*Medicago sativa*					
		Raw	3.2 ± 0.2^a	0.2 ± 0.0^a	0.2 ± 0.0^a	46.2 ± 0.5^a
		Germinated	5.4 ± 0.3^b	0.5 ± 0.0^b	0.2 ± 0.0^b	133.4 ± 10.4^b
Lentil	*Lens sculenta*					
		Raw	1.1 ± 0.1^a	0.5 ± 0.0^b	0.1 ± 0.0^b	68.6 ± 5.9^a
		Germinated	2.2 ± 0.1^b	0.7 ± 0.0^a	0.1 ± 0.0^a	217.3 ± 10.1^b
Mung bean	*Vigna radiata*					
		Raw	1.0 ± 0.1^a	N.D.	0.1 ± 0.0^a	14.3 ± 0.9^a
		Germinated	4.7 ± 0.1^b	2.5 ± 0.0^b	0.4 ± 0.0^b	166.2 ± 11.8^b
Onion	*Allium cepa*					
		Raw	0.4 ± 0.0^a	0.2 ± 0.0^a	0.2 ± 0.0^a	220.2 ± 10.3^a
		Germinated	2.3 ± 0.1^b	0.4 ± 0.0^b	0.3 ± 0.0^b	302.3 ± 23.3^b
Broccoli	*Brassica oleraceae*					
		Raw	7.3 ± 0.2^a	0.1 ± 0.0^a	0.1 ± 0.0^a	408.9 ± 38.0^a
		Germinated	16.4 ± 0.4^b	0.5 ± 0.0^b	0.3 ± 0.0^b	439.1 ± 13.8^a
Red cabbage	*Brassica oleraceae capitata rubra*					
		Raw	7.5 ± 0.4^a	0.2 ± 0.0^a	0.2 ± 0.0^a	337.6 ± 40.1^a
		Germinated	21.6 ± 0.3^b	0.5 ± 0.0^b	0.4 ± 0.0^b	857.4 ± 51.0^b
Radish	*Raphanus sativus japonicum, rambo, sinicum rosae*					
		Raw	6.7 ± 0.0^a	0.1 ± 0.0^a	0.1 ± 0.0^a	277.2 ± 7.0^a
		Germinated	20.4 ± 1.1^b	0.3 ± 0.0^b	0.4 ± 0.0^b	536.3 ± 41.8^b

Mean values within a column and vegetal sample followed by different superscript letter were significantly different at $p < 0.05$. Means ± SD ($n = 3$). N.D. no detected.

the most potent scavenging compound, as it was reported (Sharma & Bhat, 2009) (Table 2). Gallic acid and catechin showed similar AE values, although their EC_{50} exhibited differences (0.06 and 0.14, respectively) they were balanced by their T_{EC50}. Despite melatonin shows low T_{EC50} value, its antiradical efficiency is the lowest because of its relevant EC_{50}; in case of p-coumaric and kaempferol AE values were not detected. Therefore, these results showed that not only EC_{50} and ARP are needed to be determined in antioxidant compounds but also T_{EC50} to identify the power and behaviour DPPH· radical scavengers.

3.3. Total phenolic compounds

The total phenolic compounds, catechins and proanthocyanidins from raw and germinated seeds are presented in Table 3. Red cabbage and broccoli showed the highest contents of total phenolic compounds (7.5 and 7.4 mg GAE/g DW, respectively), followed by radish and alfalfa while onion exhibited the lowest levels (0.4 mg GAE/g DW). These results were similar to those reported by Xu and Chang (2007). As other bioactive compounds, the influence of processing is relevant causing general decreases on total phenolic contents (Martín-Cabrejas et al., 2009). However, germination brought about further significant increases in all edible seeds, which varied from 69% in alfalfa to five-fold in onion. These results are in agreement to those reported by earlier reports (Cevallos-Casals & Cisneros-Zevallos, 2010; Dueñas, Hernández, Estrella, & Fernández, 2009). In contrast, Khandelwal, Udipi, and Ghurgre (2010) and Swieca, Gawlik-Dziki, Kowalcyk, and Złotek (2012) detected reductions of total polyphenols in Indian pulses due to the increase of polyphenol oxidase activity and other catabolic enzymes.

As a consequence of processing there are changes that may occur in the content of phenolic families as catechins or proanthocyanidins, these changes will depend on the type of vegetable and cultivar (Bartolomé, Estrella, & Hernández, 1997). The levels of total catechins exhibited similar values in raw seeds, ranged from 0.1 mg CAE/g DW in broccoli and radish to 0.5 mg CAE/g DW in lentil. No catechin content was detected in mung bean. The presence of proanthocyanidins in raw seeds also showed low values, represented <10% of total phenols. Nevertheless, the content of catechins and proanthocyanidins was greatly affected by the processing, showing remarkable increases of these polyphenol families in all germinated seeds (until 5-fold in broccoli). Our data agree with those found by earlier authors (Bartolomé et al., 1997; Dueñas, Hernández, & Estrella, 2007; Guo, Li, Tang, & Liu, 2012). These increases could be attributed to the biochemical changes of seeds during germination, which might produce some secondary plant metabolites, such as flavonoids due to enzymatic activation (Dueñas et al., 2009). However, decreases of proanthocyanidins were reported during germination as consequence of the enzymatic hydrolysis of various components including carbohydrate, protein, fibre and lipid, as well as polyphenols (Khandelwal et al., 2010). From our results, germination promotes differences in terms of phenolic compounds that contribute to the overall antioxidant activity of plant food.

3.4. Melatonin content

Melatonin is a compound naturally present in low concentration in food, and its determination results difficult due to the amphipathic characteristic. The available scientific data of melatonin in foods has been scarce until now to make dietary intake evaluation possible (Paredes, Korkmaz, Manchester, Dun-Xian, & Reiter, 2009). Table 3 also shows the content of melatonin determined by ELISA in raw and germinated seeds. Regarding raw material, broccoli exhibited the highest melatonin content (409 pg/g DW), followed by red cabbage, radish and onion (337, 277 and 220 pg/g DW, respectively). Raw lentil, alfalfa and mung bean can hardly be detected. These results are in agreement to those found in other plant foods (Arnao & Hernández-Ruiz, 2007) and also confirm the concentration of melatonin is greatly variable in plant species.

The results demonstrated the influence of germination process on melatonin content is remarkable, showing increases in all germinated seeds. In some cases, the increases of melatonin were relevant, 3-fold in lentil and alfalfa, 2.5-fold in red cabbage and 2-fold in radish. The highest level was found in red cabbage (857 pg/g DW) followed by radish and broccoli, while onion (303 pg/g DW), lentil (217 pg/g DW), mung bean (166 pg/g DW) and alfalfa (133 pg/g DW) exhibited the lowest levels. Our data was in agreement to Zielinski, Lewczuk, Przybylska-Gornowicz, and Kozłowska (2001), who found presence of melatonin in lentil, vetch and soybean seeds as well as increases in melatonin levels during 7-day germination. We report here the presence of

Table 4
Antioxidant capacity values by different assays (DPPH, FRAP and ORAC μmol Trolox/g DW) of raw and germinated seeds.

Common name	Scientific name	Seed	DPPH (μmol Trolox/g DW)	FRAP (μmol Trolox/g DW)	ORAC (μmol Trolox/g DW)
Alfalfa	*Medicago sativa*				
		Raw	2.0 ± 0,4[a]	6.4 ± 0.2[a]	1.6 ± 0.0[a]
		Germinated	2.2 ± 0.3[b]	7.5 ± 1.0[b]	1.6 ± 0.0[b]
Lentil	*Lens sculenta*				
		Raw	2.7 ± 0.3[a]	5.3 ± 0.4[a]	0.7 ± 0.0[b]
		Germinated	2.7 ± 0.3[a]	6.6 ± 0.5[b]	1.0 ± 0.0[a]
Mung bean	*Vigna radiata*				
		Raw	1.7 ± 0.1[a]	3.9 ± 0.3[a]	0.8 ± 0.0[a]
		Germinated	3.9 ± 0.6[b]	9.9 ± 0.9[b]	0.8 ± 0.0[b]
Onion	*Allium cepa*				
		Raw	1.4 ± 0.0[a]	7.3 ± 0.4[a]	0.8 ± 0.0[a]
		Germinated	3.6 ± 0.3[b]	12.3 ± 0.2[b]	0.8 ± 0.0[b]
Broccoli	*Brassica oleraceae*				
		Raw	2.6 ± 0.0[a]	38.1 ± 2.4[a]	2.4 ± 0.0[a]
		Germinated	2.9 ± 0.0[b]	60.7 ± 2.9[b]	3.4 ± 0.0[b]
Red cabbage	*Brassica oleraceae capitata rubra*				
		Raw	2.2 ± 0.2[a]	44.5 ± 1.3[a]	2.4 ± 0.0[a]
		Germinated	4.2 ± 0.4[b]	77.1 ± 4,3[b]	3.3 ± 0.0[b]
Radish	*Raphanus sativus japonicum rambo, sinicum rosae*				
		Raw	1.4 ± 0.1[a]	30.6 ± 1.0[a]	1.5 ± 0.0[a]
		Germinated	3.7 ± 0.3[b]	77.4 ± 3.2[b]	3.3 ± 0.0[b]

Mean values within a column and vegetal sample followed by different superscript letter were significantly different at $p < 0.05$. Means ± SD ($n = 3$).

germinated seeds that can be considered as a food source of dietary melatonin. The question arises whether the amounts present in germinated seeds can be sufficient to increase the plasma level of melatonin and antioxidant activity as previously proposed by Reiter, Manchester, and Tan (2005) in walnuts and Maldonado et al. (2009) in beer. It is possible to ingest sufficient quantities of melatonin in edible plants to perhaps influence on physiological processes. Thus, considering the potent antioxidant activity of melatonin, the ingestion of germinated seeds selected by their high melatonin content, may play a role as the potential enhancement of the nutraceutical value of these seeds.

3.5. Antioxidant capacity of samples

The antioxidant activity of raw and germinated seeds was determined by DPPH, FRAP and ORAC methods (Table 4) to give a comprehensive prediction of their antioxidant efficacy. It was observed that red cabbage seeds showed the highest antioxidant capacity in all assayed methods, followed by broccoli and radish. It must be underlined that the antioxidant activity of seeds determined by different antioxidant assays gives different results (Gorinstein et al., 2005).

The antioxidant capacity of raw seeds, measured by DPPH assay, ranged from 1.4 μmol Trolox/g DW in onion and radish to 2.6 μmol Trolox/g DW in broccoli, while FRAP results ranged from 3.9 μmol Trolox/g DW in mung bean to 44.5 μmol Trolox/g DW in red cabbage and ORAC assay revealed levels that ranged from 0.7 μmol Trolox/g DW in lentil to 2.4 μmol Trolox/g DW in broccoli and red cabbage (Table 4). The different results found by these three methods (FRAP, ORAC and DPPH) can be explained by the involved mechanisms. In FRAP assay is based on electron transfer (ET) mechanism, ORAC assay uses a hydrogen atom transfer (HAT) mechanism and in case of DPPH assay, the reaction between DPPH radicals and the antioxidants can go simultaneously through an HAT and ET mechanisms (Huang, Ou, & Prior, 2005). Interestingly, the highest levels of antioxidant capacity are related to those seeds which exhibited the highest contents of phenolic compounds and melatonin. Literature data demonstrate phenolic compounds contribute to the overall antioxidant activity of the seeds (Gorinstein et al., 2005; Xu and Chang (2007)). Our results suggest that additional antioxidants as melatonin may also affect to antioxidant activity. This compound could exert a synergist effect with phenolic compounds, which may be the origin of the observed differences in the antioxidant activities of samples.

After germination process, higher antioxidant activities were exhibited in all seeds (increasing until three-fold), similar behaviour has been reported in the literature (Dueñas et al., 2007, 2009). In all assayed methods, the highest antioxidant activities were found in germinated red cabbage, radish and broccoli seeds. The maximum values were obtained by FRAP method in red cabbage and radish (77 μmol Trolox/g DW). From our results, the germinated seeds with the highest levels of polyphenols and melatonin were those exhibited the most relevant scavenging capacities.

In addition, correlation analyses between total phenolic contents, melatonin and antioxidant activities (FRAP, DPPH and ORAC assays) were performed; in all cases, high correlations between phenolic compounds and antioxidant activities, as well as melatonin and antioxidant activities ($r = 0.90–0.98$, $p < 0.01$) were shown. Relevant connection between these bioactive compounds and their antioxidant activity was also found in literature data (Gorinstein et al., 2005; Xu and Chang (2007)). Overall, the polyphenols and melatonin contents of germinated seeds were greatly affected by the processing contributing to the increase of the overall of antioxidant activity of the studied samples.

3.6. Kinetic study of samples

Finally, the DPPH radical scavenging method was applied to the evaluation of the antiradical activity of raw and germinated samples. The experiments were performed in an excess of DPPH· in order to exhaust the H-donating capacity of samples. The more rapidly the absorbance decreased, the more potent the antiradical activity of plant material in terms of hydrogen donating ability was shown (Manchester et al., 2000). For each sample at a concentration of 500 μM/mol DPPH· was tested and the percentage of DPPH· remaining at the steady state was determined in raw and germinated seeds (Fig. 2a and b). The raw seed which reacts more quickly with the DPPH was red cabbage, reaching a steady state in less than 20 min; followed by lentil at 40 min, while broccoli and radish at 60 min. The rest of samples (mung bean, alfalfa and onion) showed very slow reactions (steady state after 90 min). The Fig. 2a shows relevant antiradical activities in raw red cabbage, broccoli and radish seeds, being the percent of inhibition of the

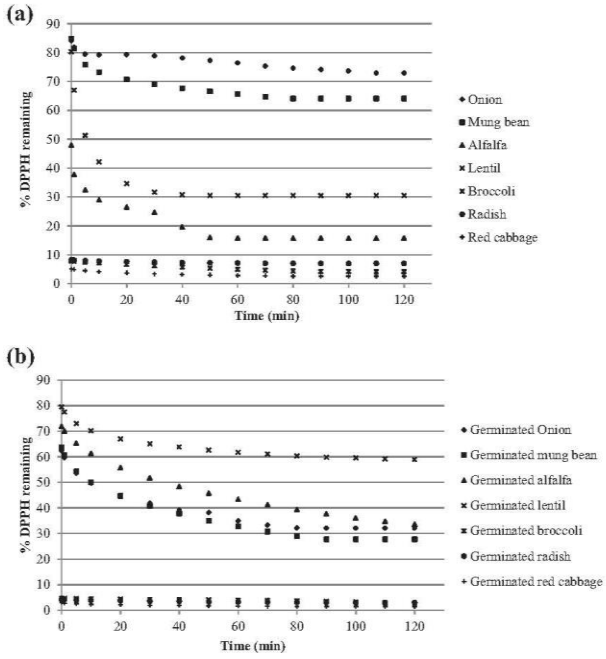

Fig. 2. Time course of scavenging of DPPH free radical by raw and germinated seeds at same solution. (500 μM/mol DPPH tested): (a) kinetic behaviour of raw seeds; (b) kinetic behaviour of germinated seeds.

DPPH· higher than 90% while onion and mung bean seeds exhibited less than 40%.

After germination process, the time of the steady state is similar to raw samples; however, in most germinated samples the inhibition percent of DPPH· was improved (Fig. 2b). This is the case of onion that exhibited a higher percent of DPPH inhibition (68%) if we compare to raw seeds (27%); and also mung bean experimented a noticeable increase from 36% to 72% of DPPH inhibition. The rest of germinated seeds showed the same behaviour, except in lentil that decreased from 70% to 41% and in alfalfa from 84 to 67% of DPPH inhibition. It can be observed that germinated red cabbage, broccoli and radish exhibited the highest antiradical activities (>97%) due to their important levels of phenolic compounds and melatonin and thus, they can offer potential health benefits.

Because the consumption of some foods requires thermal treatment and could destroy melatonin by its known stability lack, the germination process is an inexpensive and simple method of improving nutritive value. From our results, the increases of melatonin and phenolic compounds besides bioactive properties such as scavenging capacity may reveal a potential key role of germination process on the above components, as well as the possible enhancement of the functional value of these germinated seeds. The information provided in this study is valuable for the selection of edible germinated seed species to develop functional foods. Thus, it may be considered the incorporation of red cabbage, radish and broccoli germinated seeds into the diet to offer potential health benefits.

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Publicación III. Efecto de la iluminación en el contenido de melatonina, compuestos fenólicos y actividad antioxidante durante la germinación de lentejas (*Lens culinaris* L.) y judías (*Phaseolus vulgaris* L.)

Yolanda Aguilera, Rosa Liébana, Teresa Herrera, Miguel Rebollo, Carlos Sanchez-Puelles, Vanesa Benítez, María Ángeles Martin-Cabrejas. Effect of illumination on the content of melatonin, phenolic compounds and antioxidant activity during germination of lentils (*Lens culinaris* L.) and kidney beans (*Phaseolus vulgaris* L.).*J. Agric. Food Chem*. **2014**, *52*, 10736-10743

RESUMEN

El estudio muestra el efecto de dos diferentes condiciones de iluminación durante la germinación (12h luz/12 h oscuridad vs. 24 h oscuridad) en lentejas (*Lens culinaris* L.) y judías (*Phaseolus vulgaris* L.) sobre el contenido de melatonina y compuestos fenólicos, además de la actividad antioxidante. La germinación produce un incremento relativo en el contenido de melatonina y sobre la actividad antioxidante, mientras que los niveles de compuestos fenólicos se reducen. Se observa un elevado contenido de melatonina tras 6 días de germinación bajo 24 h de oscuridad para ambas leguminosas. Las semillas de leguminosas germinadas con mejores niveles de melatonina desempeñan un papel protector contra los radicales libres. Por lo tanto, teniendo en cuenta la actividad antioxidante de la melatonina, estos germinados podrían ser incluidos en la dieta, siendo una estrategia alimentaria preventiva en la lucha contra las enfermedades crónicas a través de la dieta.

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Effect of Illumination on the Content of Melatonin, Phenolic Compounds, and Antioxidant Activity During Germination of Lentils (*Lens culinaris* L.) and Kidney Beans (*Phaseolus vulgaris* L.)

Yolanda Aguilera, Rosa Liébana, Teresa Herrera, Miguel Rebollo-Hernanz, Carlos Sanchez-Puelles, Vanesa Benítez, and María A. Martín-Cabrejas*

Instituto de Investigación de Ciencias de la Alimentación (CIAL), Facultad de Ciencias, Universidad Autónoma de Madrid, C/Nicolás Cabrera 9, 28049 Madrid, Spain

ABSTRACT: This study reports the effects of two different illumination conditions during germination (12 h light/12 h dark vs 24 h dark) in lentils (*Lens culinaris* L.) and kidney beans (*Phaseolus vulgaris* L.) on the content of melatonin and phenolic compounds, as well as the antioxidant activity. Germination led to relative increase in melatonin content and significant antioxidant activity, while the content of phenolic compounds decreased. The highest melatonin content was obtained after 6 days of germination under 24 h dark for both legumes. These germinated legume seeds with improved levels of melatonin might play a protective role against free radicals. Thus, considering the potent antioxidant activity of melatonin, these sprouts can be consumed as direct foods and be offered as preventive food strategies in combating chronic diseases through the diet.

KEYWORDS: *melatonin, phenolic compounds, antioxidant capacity, germination, legumes, sprouts*

INTRODUCTION

Lentils and beans are the most widely consumed legume seeds by a large part of the world's human population. Many studies have been carried out to determine the role of legumes as preventive agents in the diet of vulnerable populations (diabetes, obesity, cardiovascular diseases, etc.). However, the utilization of legumes is limited by the presence of antinutrient compounds, such as protease inhibitors, nonprotein amino acids, lectins, saponins, and flatulence compounds and, hence, they should be processed before consumption. In this regard, germination has been identified as a low-cost technology to improve the quality of legumes, by enhancing their digestibility and increasing their content of bioactive compounds.[1−5] Phenolic compounds have been widely studied as antioxidants due to their ability in querching free radicals contributing to total antioxidant capacity and their protection role against highly prevalent diseases.[6,7] Recently, the attenuation of oxidative stress by germinated food consumption is reached through increases in antioxidant levels in plasma and antioxidant enzyme activity in different animal tissues.[8] Increases of bioactive compounds during legume germination vary greatly depending on the plant species, seed varieties, and germination.[9,10] So far, little information has been reported about the effects of germination on melatonin content, a hormone that has been described as an effective free scavenger showing antioxidant properties against lipid peroxidation due to its amphipathic nature.[11]

Melatonin (*N*-acetyl-5-methoxytryptamine), an indolamine, is a ubiquitous and highly conserved molecule occurring in animals (vertebrates and invertebrates), bacteria, mono- and multicellular algae, and plants. Melatonin plays a key role in plant physiology as a mechanism to increase the survival and perpetuation of species as circadian regulator, cytoprotector, and regulator of plant growth.[12,13] Melatonin might be involved in mechanisms of preservation of chlorophyll, promotion of photosynthesis, and stimulation of root development. In mammals, besides acting as a regulator of circadian and seasonal rhythms, melatonin is involved in antioxidative systems, vascular tone, and inhibition of infections and tumors.[14−16] Melatonin has been detected in plant foods, including cereals, vegetables and fruits, and roots.[17,18] Indeed, plant tissues seem to contain much higher melatonin levels than those observed in animals.[15] Melatonin concentration has been shown to vary from a few picograms to micrograms per gram of plant material.[19] Nowadays, the beneficial human health effects of melatonin derived from the consumption of plant foods are being considered.[20] In the last years, attention was also paid to incorporating plant foods with high melatonin levels as a dietary supplement to increase its blood plasma levels in humans and, consequently, its scavenging and antioxidant action.[21] Thus, we have focused this work on the impact of germination conditions in the melatonin and phenolic compound content, as well as the antioxidant activity of legumes.

The objectives of the present study were to examine melatonin content under two different illumination germination conditions (12 h light/12 h dark and 24 h dark) for 3, 6, and 8 days in two common legumes (*Lens culinaris* L. and *Phaseolus vulgaris* L.). Moreover, the content of phenolic compounds was determined besides melatonin to evaluate their influence on the antioxidant activity as a strategy to design novel foods for improving consumers' health.

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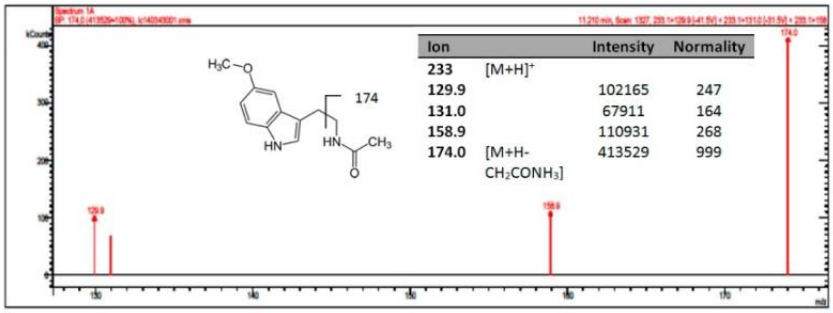

Figure 1. Melatonin spectrum and mass accuracy.

MATERIALS AND METHODS

Plant Materials. Two varieties of common legumes: lentil (*Lens culinaris* L., var. *Salmantina*) and kidney bean (*Phaseolus vulgaris* L., var. *Pinta*) were provided by Institute of Food Science, Technology and Nutrition (CSIC, Madrid). They were stored in dark and dry conditions under refrigeration.

Processing Conditions. Three batches of seeds (20 g of lentils and 50 g of beans) were sterilized in 1% NaClO solution for 30 min to reduce bacterial and fungal growth. Seeds were washed three times with distilled water and then soaked in distilled water for 5 h for seed hydration (ratio 1 g:20 mL, seed:water). They were germinated in an incubator (Construcciones Frigoríficas, Confri, S.L.) at 20 °C and 80% RH, illumination conditions of photoperiodic cycle (12 h light/12 h dark) and darkness (24 h dark) for 3, 6, and 8 days. Lentil and kidney bean sprouts were withdrawn, frozen in N_2, freeze-dried, and milled. Flours were passed through a 50-μm sieve and stored at 4−6 °C for further analysis.

Melatonin Extraction and Quantification by HPLC-MS/MS. Extraction was conducted according to Manchester et al.[22] and Ansari et al.[23] with some modifications. Briefly, lentil and kidney bean flours (2 g) were extracted with 10 mL of methanol (MeOH). Extracts were stored for 16 h at 4 °C in darkness. After a brief centrifugation at 2000*g* for 15 min at room temperature; the supernatants were filtered under vacuum by a filter (11 μm, Whatman) and they were evaporated to dryness under N_2 (g). The residues were resuspended in 2 mL of water, after a mild ultrasonic treatment for 2 min at room temperature; the reconstituted extracts were filtered using the solid phase extraction (SPE, cartridge C-18, Waters). The extracts obtained were evaporated to dryness by use of evaporator centrifuge (Speed Vac SC 200, Savant, USA). The residues were dissolved in MeOH/H_2O (80:20; v/v) + 0.1% formic acid. Melatonin was determined by HPLC-ESI-MS/MS triple quadrupole (Varian 1200L with API-ES between 10 and 1500 Da range mass). Melatonin was recorded using multiple reaction monitoring (MRM) mode by selecting ion 233 ($M + H^+$) at the first quadrupole (Q1), fragmented in Q2 using Ar and analyzing resulting ion m/z 130, 131, 159, and 174 at Q3, measuring at 174 (Figure 1). The system consisted of two pumps, Varian Prostar 210, and an Intensity Trio C18 (150 mm × 2.0 mm ID, S-3 μm, 12 nm BRYTC18032150) (Bruker) column, at a flow rate of 0.2 mL/min. Acetonitrile containing 0.1% formic acid (solvent A) and 0.1% formic acid in H_2O (solvent B) as mobile phase were pumped with the following gradient: 5% solvent A (0−4 min), 5−100% solvent A (4−15 min), 100% solvent A (15−18 min), and 100−5% solvent A (18−25 min) to recover initial conditions. The injection volume was 50 μL. Quantification was performed using external standard method, using pure melatonin (Sigma-Aldrich Química, Spain) dissolved in MeOH/H_2O (80:20; v/v) containing 0.1% formic acid, showing good regression coefficient (R^2 = 0.9989). Samples and standard solutions were analyzed in triplicate. Melatonin content was expressed as ng/g dry weight (DW).

Phenolic Compounds Extraction and Determination. Extraction of phenolic compounds was carried out following the Gou et al.[24] method with some modifications. Germinated flours (2 g) were blended using 30 mL of 80% chilled acetone (1:2, w/v) for 5 min and vortex for 10 min. The homogenates were then centrifuged at 2054*g* for 5 min. Supernatants were collected, and the extraction was repeated three times. All the supernatants were pooled and evaporated until 10% of the supernatants remained. The soluble phytochemical extracts were brought to 10 mL in water and were kept at −40 °C until analysis. For the extractions of bound phytochemicals, the residues from above soluble free extraction were flushed with N_2 and hydrolyzed directly with 20 mL of 4 M NaOH at room temperature for 1 h with shaking. The mixture was acidified to pH 2 with concentrated HCl, centrifuged at 2958*g* for 5 min, and extracted three times with ethyl acetate. The ethyl acetate fractions were evaporated to dryness, and were reconstituted into 1.5 mL of methanol and stored at −40 °C until analysis.

The phenolic compounds were determined by Folin−Ciocalteu colorimetric method according to Singleton et al.[25] using gallic acid (GAE) as standard. Phenolic compounds were expressed as mg GAE/100 g DW.

Oxygen Radical Absorbing Capacity (ORAC) Assay. The above methanol extracts were used for determining the radical absorbance activity by ORAC method using fluorescein as a fluorescence probe.[26] Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained fluorescein (70 nM), 2,2-azobis (2-methyl-propionamidine)-dihydrochloride (12 mM), and antioxidant standard (Trolox [10−80 μM] or sample extracts). A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) was used to read fluorescence at wavelengths of 485 nm excitation and 520 nm emission. The equipment was controlled by the Fluostar Galaxy software version (4.31-0) for fluorescence measurement. Black 96-well untreated microplates (PS Balck, Porvair, Leatherhead, UK) were used. The plate was automatically shaken before preincubation, and the fluorescence was recorded every minute for 80 min. All reaction mixtures were prepared in duplicate and at least 3 independent runs were performed for each sample. Fluorescence measurements were normalized to the oxidation control (phosphate buffer) and stability control (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} f_i / f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \text{AUC antioxidant} - \text{AUC blank}$$

The net AUC was plotted against the antioxidant concentration, and the regression equation of the curve was calculated. The ORAC

value was obtained by dividing the slope of the latter curve between the slopes of the Trolox curve obtained in the same assay. Final ORAC values were expressed as μmol of Trolox equivalents/g of dry sample (μmol TE/g DW).

Statistical Analysis. Each sample was analyzed in triplicate. Data were expressed as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance (ANOVA) and posthoc Duncan test. Differences were considered to be significant at $p < 0.05$. The statistical analysis was performed by SPSS 17.0.

RESULTS AND DISCUSSION

Germination Process. The changes in biomass and percents of germination under different germination conditions are shown in Table 1. Legume biomass increased from 111% to 162% and lentil sprouts showed higher levels than bean sprouts at the same germination conditions. The highest rise was reached in lentil under eighth day 12 h light/12 h dark. The success of this processing exhibited good viability for both legumes, reaching 100% in case of bean sprouts at 12 h light/12 h dark on the eighth germination day. However, lentil exhibited lower germination percentage on the eighth day due to fungal growth. The parameter of development of radicle was different not only dependent on the kind of leguminous seeds, but also on process conditions (i.e., the presence of light and germination time). The length of bean radicle was higher than of lentils, irrespective of light conditions and germination time. These differences could be attributed to changes in bioactive compounds (melatonin) that promote vegetative plant growth.[12]

Table 1. Changes in Seed/Seedlings Biomass and Percent of Germination at Different Illumination Conditions of Germination[a]

illumination conditions of germination	germination time (day)	% increase in fresh weight of seeds/ seedlings	% germination	development of radicle (cm)
		Lentil		
12 h light/ 12 h dark	3	137 ± 8 a	92 ± 8 b	1.4 ± 0.1 a
	6	141 ± 9 a	58 ± 5 a	2.6 ± 0.1 b
	8	162 ± 5 b	59 ± 5 a	4.5 ± 0.2 c
24 h dark	3	138 ± 5 a	83 ± 7 b	1.4 ± 0.1 a
	6	150 ± 6 b	83 ± 7 b	4.1 ± 0.2 c
	8	156 ± 10 b	65 ± 6 a	7.3 ± 0.2 d
		Bean		
12 h light/ 12 h dark	3	104 ± 6 a	47 ± 3 a	1.0 ± 0.1 a
	6	126 ± 9 b	84 ± 5 b	3.7 ± 0.2 b
	8	133 ± 9 c	100 ± 4 c	6.1 ± 0.4 d
24 h dark	3	90 ± 7 a	46 ± 4 a	1.9 ± 0.1 a
	6	104 ± 8 a	89 ± 5 b	5.6 ± 0.2 c
	8	111 ± 9 a	96 ± 5 b	10.6 ± 0.6 e

[a]Results are reported as mean ± SD ($n = 3$). Mean values of each row of different illumination conditions followed by different superscript letter significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

Melatonin. Different methods have been developed for melatonin identification in plant extracts. Melatonin was detected in raw lentil and kidney beans varieties studied, and chromatograms are shown in Figure 2. From the results, kidney beans exhibited higher melatonin content than lentils (1.0 and 0.4 ng/g DW, respectively). These data explain the different elongation showed by legume sprouts that could be due to melatonin levels. This molecule exhibits auxinic activity in a way similar to indole-3-acetic acid (IAA).[12] In comparison to other plant foods, the results obtained were higher than those reported in other seeds such as sweet corn and rice,[27] indicating a good dietary melatonin source. The available scientific data of melatonin in foods are scarce because only a few food items have been analyzed. Melatonin levels are greatly variable among plant species, apart from seeds, fruits (12−36 pg/g WW), roots protected from light such as carrots and onions, or processed foods such as fermented drinks (0.1−35 ng/mL) and olive oil (108 pg/mL) are also natural sources of melatonin.[19]

Germination brought about a noticeable increase in melatonin ($p < 0.05$) and this effect was time-dependent in all seeds studied (Figure 3). Reports indicated higher melatonin levels at complete darkness[28] and relative increases in cucumber and red cabbage germinated seeds.[29,30] To our knowledge, the present work is the first report on the melatonin content of lentil and bean sprouts to improve the content of this bioactive compound. In this sense, melatonin content increased significantly in lentil sprouts during both germination processes, showing similar values under 12 h light/12 h dark and 24 h dark conditions (Figure 3a). In both illumination conditions, the highest contents were shown on the sixth germination day (2.3 and 2.5 ng/g DW under 12 h light/12 h dark and 24 h dark, respectively). After the sixth germination day, the levels of melatonin decreased in lentil sprouts, being more pronounced under 24 h dark (72% of decrease with respect to the sixth germination day). Similar behavior was found in bean sprouts (Figure 3b). On the sixth processing day, germination significantly influenced melatonin accumulation in both germination conditions, up to 2.4-fold at 12 h light/12 h dark and more accentuated, up to 9.4-fold at 24 h dark conditions. Finally, it is worth emphasizing that a sharp melatonin decrease (94%) from the sixth to eighth germination day was observed at 24 h darkness (0.6 ng/g DW) and less accentuated (38% of decrease) at 12 h light/12 h dark (1.5 ng/g DW). Thus, germination in darkness for 8 days did not cause melatonin increase either in lentils or beans. Our data were in agreement with those of Zielinski et al.,[31] who found contents of melatonin in lentil, vetch, and soybean seeds, as well as increases in melatonin during 7 days of germination. Hence, melatonin content in lentil showed maximum levels at sixth germination day either at 12 or 24 h dark, while melatonin content in bean reached the highest content at 24 h dark. From our results, melatonin was mainly influenced by photoperiods; darkness regimes led to more of this bioactive compound in kidney bean and these results were in agreement with those of Murch et al.[27]

The melatonin originated from these sprouts may play a role as a health promoting substance with clear antioxidant and anti-inflammatory properties; its genomic effects, and its capacity to modulate mitochondrial homeostasis, are linked to the redox status of cells and tissues.[16] From our results, the sprout intake, especially germinated beans, could be considered as a food source of dietary melatonin.

Phenolic Compounds. The free, bound (cell wall-associated), and total phenolic compounds (TPC) of lentil and bean sprouts are shown in Figure 4. TPC in raw lentil and bean seeds were 488 and 379 mg GAE/100 g DW, respectively. These legume seeds seem to be a good source of phenolic compounds and our results are within the range of previously reported data.[32−34] The predominant phenolic compounds in lentils and beans are catechins and procyanidins;[35,36] however, the phenolic composition of legume seeds may vary among

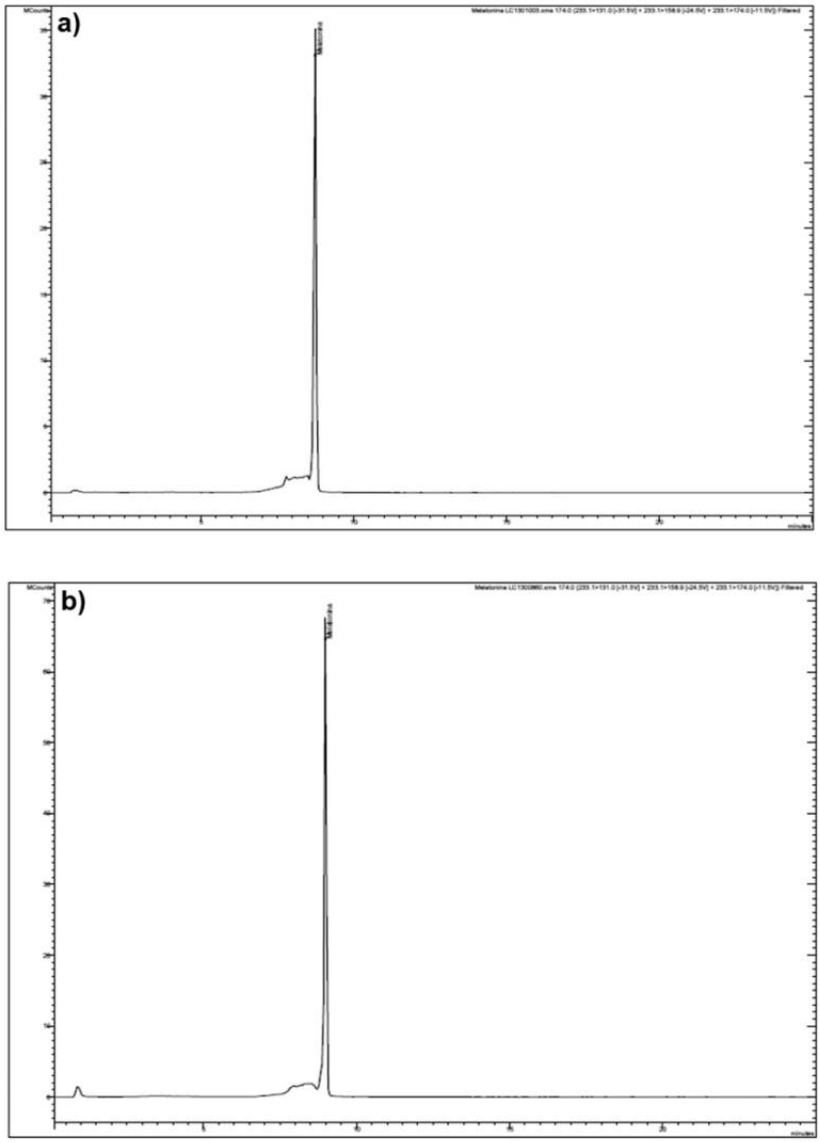

Figure 2. HPLC-MS/MS chromatograms of raw lentil (a) and raw kidney bean (b).

cultivars and genotypes. Differences among them may be attributed to differences in the color of the seed coat as has been reported in the literature,[32] lentil was darker than kidney bean. Lentil seeds showed 88% of free phenolic contents with respect to TPC (Figure 4a), while kidney bean seeds exhibited a higher percentage of free phenols (92%) and consequently, a lower percentage of bound phenols (8%) (Figure 4b). These results are in agreement with those found in other legume seeds.[24,37]

Our results exhibited that TPC decreased dramatically in lentil sprouts at both darkness germination conditions (12 and 24 h dark), while kidney bean sprouts exhibited relevant decreases at 12 h light/12 h dark but no relevant changes at 24 h dark germination conditions. These results are in agreement with the findings of Khandelwal et al.[32] and Troszyńska et al.[38]

With respect to lentil, decreases of phenolic compounds (total, free, and bound forms) were initiated at the third germination day and maintained during germination conditions

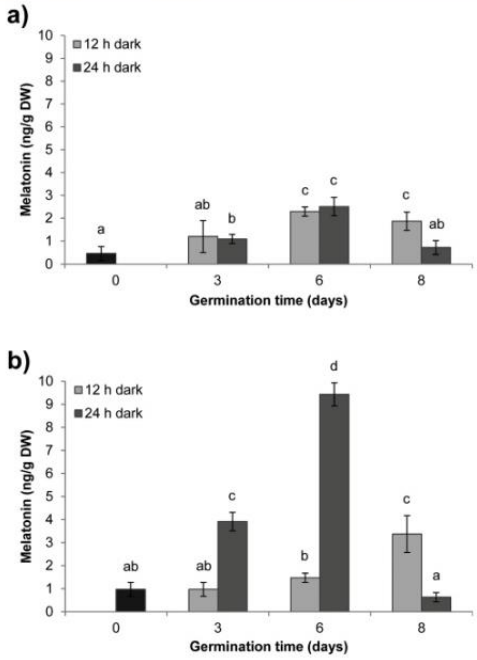

Figure 3. Melatonin content in raw and germinated lentils (a) and kidney beans (b).

(12 and 24 h dark) (Figure 4a). The germination process of 12 h light/12 h dark reduces significantly up to 72% with respect to raw lentil on the sixth germination day, but any further process did not exhibit any significant changes ($p < 0.05$). In this sense, the concentration of free phenolic acids showed the maximum decrease on the sixth germination day (from 450 to 124 mg GAE/100 g DW). Decreasing tendency was also observed in bound phenols, being more accentuated on the third germination day (from 38 to 5 mg GAE/100 g DW). Regarding 24 h dark lentil germination, a similar trend was observed but lower reduction in TPC was detected (50% of decrease at the sixth germination respect to control). This may be due to the relative amounts of free phenolic compounds present in these sprouts and, also, the significant increases of bound phenolics (up to 1.6-fold respect to raw lentil seed) exhibited under 24 h darkness (Figure 4a). Although this fraction is hardly noticeable in both legumes, bound phenols are more likely glycoside flavonols, subjected to the action of colonic microflora, releasing aglycones that might be absorbed in a lesser extent and, probably, degraded to simpler phenolic derivatives and thus, they may play a special role on health.[39]

Concerning kidney beans, 12 h light/12 h dark led to a TPC reduction in a time-dependent manner (Figure 4b). The contents of free phenolic under 12 h light/12 h dark exhibited decreases (70% reduction at the third germination day as compared to raw bean) and contributed up to 93% of TPC of bean sprouts. Bean sprouts at 24 h dark exhibited an increase of TPC due to the increases either of free or bound phenol forms but these were not significant ($p < 0.05$) in a time-dependent manner. A similar trend was also observed in germinated black

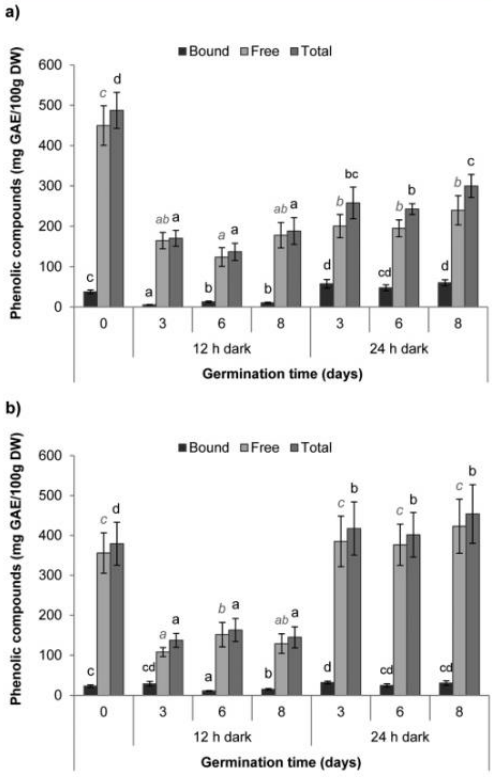

Figure 4. Bound, free, and total phenolic compounds in raw and germinated lentils (a) and kidney beans (b).

beans (*Phaseolus vulgaris* L.) with no significant difference of phenolic contents among them.[34] However, decreases in phenolic contents of legumes following germination have been reported by several authors.[32,40] These decreases may be attributed to the increases in the activity of enzymes responsible for the oxidation of endogenous phenolic compounds and other catabolic enzymes in raw and processed legumes. During germination, enzymes are activated, resulting in the hydrolysis of various components, including carbohydrate, protein, fiber, and lipid, as well as phenolic compounds.[32] Thus, the phytochemical quality of sprouts depends on many factors such as legume cultivar and germination conditions. This means that optimum germination conditions need to be defined for legume seeds to improve the functional quality of the sprout.

Antioxidant Activity. The antioxidant capacities of lentil and bean seeds (Figure 5) were similar (20.2 and 24.0 μmol TE/g DW, respectively). The results obtained were lower than those reported by Xu et al.[41] and Aguilera et al.,[35] but similar to those of Xu and Chang[37] and Guajardo-Flores et al.[34] in common beans. In general, germination brought about an enhancement of the antioxidant potential of legumes, in agreement with other studies.[7,10,34,42] Germination time directly affected the antioxidant activity of lentil and bean

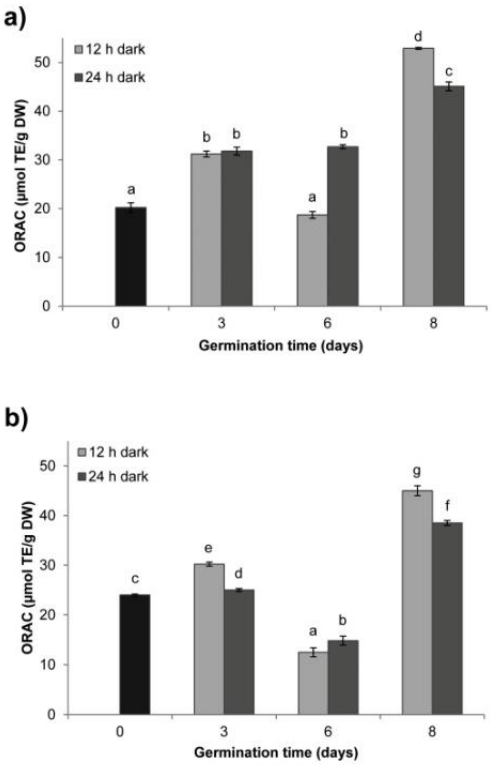

Figure 5. Antioxidant activity in raw and germinated lentils (a) and kidney beans (b).

sprouts. The antioxidant activity of both sprouts increased with longer germination time ($p < 0.05$), except at day 6. Among legume seeds, antioxidant activity also varied following different germination conditions. ORAC values were up to 2.4- and 2.2-fold higher after sprouting on the eighth germination day at 12 and 24 h dark for lentil sprouts, respectively. In general, lower increases were detected in bean sprouts (1.6- and 1.9-fold on the sixth germination day at 12 and 24 h dark, respectively). In addition, it should be pointed out that different light conditions during germination significantly influenced the antioxidant capacity of sprouts.[43] Among all of the sprouted legumes tested, it was noted that both samples germinated on the sixth germination day under 12 and 24 h dark showed the lowest antioxidant activities, with values ranged from 14.8 to 18.9 μmol TE/g DW. Interestingly, these samples corresponded to those exhibiting the lowest phenolic compound levels but the maximum contents of melatonin. In general, the antioxidant properties of melatonin and its ability to scavenge several radical species as part of its bioactivity have been described in the literature.[44,45] Melatonin has been found to neutralize the most toxic oxidizing agents generated within cells, such as hydroxyl radical (·OH) and peroxynitrite anion (ONOO−). In addition, melatonin reportedly scavenges singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO·), and hypochlorous acid (HClO).[46]

Finally, after the eighth germination day, a significant increase of antioxidant activity was also detected for both legume sprouts as mentioned before. Thus, this effect could be attributed to higher accumulation of compounds as melatonin degradation with radical scavenging activity. Longer application of germination resulted in further increment of antioxidant capacity, showing major ability of sprouts to prevent oxidative stress on the eighth germination day. The pronounced values of antioxidant capacity on day eight have an inverse correlation with values of melatonin in sprouts ($r = 0.61-0.75$, $p < 0.01$). This behavior may result from the indirect antioxidant properties of melatonin, as the activation of several antioxidant enzymatic cascades, including glutathione peroxidase, superoxide dismutase, and glutathione reductase, and the accumulation of degradation products, highly effective scavengers (AFMK, AMK, and cyclic 3-hydroxymelatonin),[46,47] bringing about the increased antioxidant capacity levels of bean sprouts on the eighth germination day.[48] Thus, the legume sprouts obtained in this study are valuable sources of natural antioxidants that most likely could positively influence the overall antioxidant status in humans.

Germination led to decreases in phenolic compounds and relative improvements in melatonin levels, bringing about significant increases of antioxidant activity in lentil and bean sprouts. Optimal germination conditions for melatonin levels seemed to be under 24 h dark at the sixth germination day for both sprouts, even though antioxidant activity increased on the eighth day. These germinated legume seeds with improved levels of melatonin might play a role because of the potential enhancement of the nutraceutical value of these seeds. Thus, considering the potent antioxidant activity of melatonin, these sprouts can be consumed as direct foods or adopted in the future consumer's food practices and be offered as preventive food strategies in combating chronic diseases through the diet.

AUTHOR INFORMATION

Corresponding Author

*E-mail: maria.martin@uam.es. Tel.: 0034 91 497 8678. Fax: 0034 91 497 3826.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AFMK, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine; AMK, N(1)-acetyl-5-methoxykynuramine; DW, Dry weight; GAE, Gallic acid equivalents; HPLC-MS/MS, High-performance liquid chromatography tandem mass spectrometry; MRM, Multiple reaction monitoring; TE, Trolox equivalents; TPC, Total phenolic compounds; WW, Wet weight

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Publicación IV. Impacto del enriquecimiento de melatonina durante la germinación sobre los compuestos bioactivos y la actividad antioxidante

Yolanda Aguilera, Teresa Herrera, Rosa Liébana, Miguel Rebollo-Hernanz, Carlos Sánchez-Puelles, María A. Martín-Cabrejas. Impact of melatonin enrichment during germination of legumes on bioactive compounds and antioxidant activity.*J. Agric. Food Chem*. **2015**, *63*, 7967-7974

RESUMEN

En este estudio se evalúa el impacto del riego con melatonina enriquecida sobre la germinación de lentejas (*Lens culinaris* L.) y judías (*Phaseolus vulgaris* L.). Los niveles de melatonina en germinados de lenteja y judía medidos por HPLC-MS/MS fueron más importantes que los encontrados en otras legumbres, siendo mayores en los germinados de lenteja (1090 ng/g m.s.) que en judía (529 ng/g m.s.). Las condiciones en las que se lleva este proceso de germinación promueven un incremento significativo en el desarrollo de radículas en comparación con la germinación tradicional. Asimismo, se observó un descenso menos acusado durante la germinación de los compuestos fenólicos que los observados previamente (germinados de lenteja 394 mg (GAE)/100 g m.s.), probablemente debido al efecto protector de la melatonina. La actividad antioxidante (ensayo de capacidad de absorción de radicales de oxígeno) se incrementó en estos germinados, alcanzando 85 y 56 µmol Trolox equivalentes /g m.s. en lentejas y judías, respectivamente. Por lo tanto, los germinados enriquecidos de melatonina exhiben un potente carácter antioxidante que podrían ser usados como herramienta nutricional para prevenir enfermedades crónicas y enfermedades relacionadas con la edad.

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Impact of Melatonin Enrichment during Germination of Legumes on Bioactive Compounds and Antioxidant Activity

Yolanda Aguilera, Teresa Herrera, Rosa Liébana, Miguel Rebollo-Hernanz, Carlos Sanchez-Puelles, and María A. Martín-Cabrejas*

Instituto de Investigación de Ciencias de la Alimentación (CIAL), Facultad de Ciencias, Universidad Autónoma de Madrid, C/Nicolás Cabrera 9, 28049 Madrid, Spain

ABSTRACT: This study assesses the impact of melatonin enriched watering on the germination of lentils (*Lens culinaris* L.) and kidney beans (*Phaseolus vulgaris* L.). The melatonin levels in lentil and bean sprouts measured by HPLC-MS/MS were more important than those found in other legumes and sprouts, being higher in lentil (1090 ng/g) than in kidney bean (529 ng/g) sprouts. This alternative germination promoted a significant increase of the development of radicles in comparison with the traditional germination. The decreases in the phenolic load were less accentuated than previously observed (lentil sprouts displayed 394 mg gallic acid equivalents (GAE)/100 g of dry weight (DW)), probably due to the protective effect of melatonin. The antioxidant capacity (oxygen radical absorbing capacity assay) increased in these sprouts, reaching 85 and 56 μmol of Trolox equivalents/g DW in lentils and beans, respectively. Hence, the melatonin-enriched foods exhibited potent free radical scavenger and antioxidant functions that may be used as a nutritional strategy to alleviate and prevent chronic and age-related diseases.

KEYWORDS: *exogenous melatonin, germination, melatonin, phenolic compounds, antioxidant capacity, legumes*

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) has long been known as an animal neurohormone, produced in the pineal gland and involved in daily cycles. However, this indolamine has been found in numerous organisms.[1−3] In mammals, melatonin shows antioxidant and free radical scavenger activities against many radical species,[4,5] up-regulating several antioxidative enzymes[6,7] and thereby protecting cells from possible oxidative damage. In plants, its antioxidant function also is supported by strong experimental evidence.[8,9] Thus, melatonin protects plants against oxidative stress, reduces damage to macromolecules, and also up-regulates certain protective enzymes in the senescence process.[2,10,11] Melatonin also plays a role in growth promotion, coordination of photoperiodic responses, regulation of plant reproductive physiology, and defense of plant cells against apoptosis induced by harsh environmental conditions.[11,12] Evidence also suggests that melatonin exhibits the ability to increase crop production.[13,14] Exogenous melatonin in water provided cellular protection during seed growth and senescence, enhancing tolerance and survival against environmental stress conditions as well as promoting root development and increasing germination rate.[10,12,13,15−17]

Legume grains, besides being a great source of nutrients, contain different types of antioxidants and compounds beneficial to human health.[18,19] Edible legume seeds and sprouts are good sources of phytochemicals that reduce the risk of developing oxidative stress-associated diseases and age-related functional decline.[20,21] Recently, germinated food consumption has been suggested to attenuate oxidative stress through increases of antioxidant capacity in plasma and antioxidant enzyme activities.[22] Germination is a cheap and effective way to improve the nutritional value and quality of legumes by enhancing their digestibility and increasing their load of bioactive compounds.[23−26] Sprouting promotes significant changes on legume phenolic composition, mainly due to endogenous enzyme activation and complex metabolism of seeds during this process.[27,28] Phenolic compounds have been extensively studied due to their antioxidant and free radical scavenging properties; they have beneficial properties in humans or animals, such as antihypertensive and antibacterial actions and anticarcinogenic properties in vitro and animal models.[18,22,27] Although phenolic compounds represent the archetype of compounds that promote health, melatonin is relevant due to its biological activities and its excellent bioavailability.[29,30] Several studies have recently shown how germination also induces an enhancement of melatonin content in seeds and sprouts,[31−33] contributing thereby to the beneficial properties of germinated seeds.

Currently, the incorporation of melatonin as dietary supplement has received increased attention. Melatonin intake can increase its blood plasma levels in humans and, consequently, produce its beneficial scavenging and antioxidant action.[34] The European Food Safety Authority (EFSA) has recently accepted a health claim in relation to melatonin and reduction of sleep onset latency, recommending the consumption of 1 mg of melatonin close to bedtime.[35] However, there is not enough scientific data on its content in foods to evaluate its dietary intake.

The aim of the present work was to study the impact of melatonin enriched watering on the germination of two common legumes (*Lens culinaris* L. and *Phaseolus vulgaris* L.) quantifying the absorption of melatonin, the phenolic load, and

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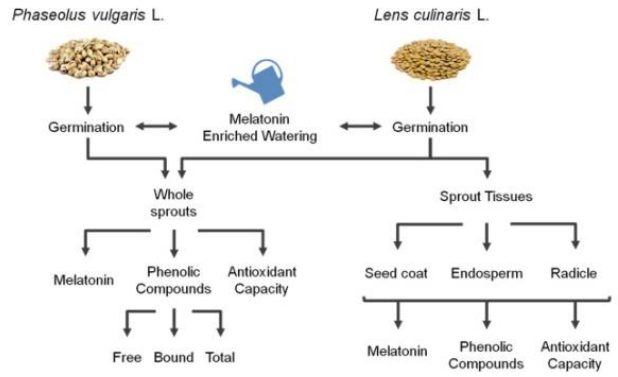

Figure 1. Illustrative diagram of the experimental design.

the effect on the antioxidant capacity in both sprouts. In addition, a sensory analysis was carried out on hummus of lentil sprouts to evaluate the influence of adding exogenous melatonin during their germination, in order to assess the potential legume sprout-based food for improving consumer's health.

MATERIALS AND METHODS

Plant Materials. Two varieties of common legumes, lentil (*Lens culinaris* L., var. *Salmantina*) and kidney bean (*Phaseolus vulgaris* L., var. *Pinta*), were provided by Institute of Food Science, Technology and Nutrition (CSIC, Spain) and stored under refrigeration conditions.

Processing Conditions. Three batches of seeds (20 g of lentils and 50 g of kidney beans) were sterilized in 1% NaClO solution for 30 min to reduce bacterial and fungal growth. Seeds were washed three times with distilled water until neutral pH and then soaked in a 20 μM melatonin solution for 5 h for seed hydration (ratio 1:20, m/v). Seeds were germinated for 3, 6, 8, and 10 days in an incubator (Construcciones Frigoríficas, Confri, S. L.) at 20 °C, 80% RH, and darkness. Seeds were maintained wet by capillarity using a 20 μM melatonin solution. Lentil and kidney bean samples were withdrawn, frozen in N_2, freeze-dried, and milled. Flours were passed through a 50 μm sieve and stored at 4−6 °C for further analysis. In addition, lentil sprouts were separated into different tissues as seed coat, endosperm, and radicle. These samples were also frozen in N_2, freeze-dried, and milled. Flours were passed through a 50 μm sieve and stored at 4−6 °C for further analysis. The experimental design is illustrated in Figure 1.

Melatonin Extraction and Quantification by HPLC-MS/MS. The extraction procedure was carried out according to Aguilera et al.[32] Briefly, lentil and kidney bean flours (2 g) were extracted with 10 mL of methanol (MeOH) for 16 h at 4 °C in darkness. After a brief centrifugation at 2000*g* for 15 min at room temperature, the supernatants were filtered under vacuum by a filter (11 μm, Whatman) and evaporated to dryness under N_2 (g). The residues were resuspended in 2 mL of water, after a mild ultrasonic treatment for 2 min at room temperature. The reconstituted extracts were filtered using solid phase extraction (SPE, cartridge C-18, Waters) as follows: first, a conditioning step was carried out with 1 mL of MeOH and 1 mL of water to activate the column, followed by sample application (1 mL of extract) and washing step with 2 mL of MeOH 10%; finally, 1 mL of MeOH was used for the elution step. The extracts obtained were evaporated to dryness by use of an evaporator centrifuge (Speed Vac SC 200, Savant, USA). The residues were dissolved in MeOH/H_2O (80:20, v/v) + 0.1% formic acid. Melatonin was determined by HPLC-ESI-MS/MS triple quadrupole (Varian 1200L with API-ES between 10 and 1500 Da range mass). The system consisted of two pumps, Varian Prostar 210 and an Intensity Trio C18 (150 mm × 2.0 mm ID, S-3 μm, 12 nm BRYTC18032150; Bruker) column, at a flow rate of 0.2 mL/min. Acetonitrile containing 0.1% formic acid (solvent A) and 0.1% formic acid in H_2O (solvent B) as mobile phase were pumped with the following gradient: 5% solvent A (0−4 min), 5−100% solvent A (4−15 min), 100% solvent A (15−18 min), and 100−5% solvent A (18−25 min) to recover initial conditions. The injection volume was 50 μL. Quantification was performed using the external standard method, using pure melatonin (Sigma-Aldrich Química, Spain) dissolved in MeOH/H_2O (80:20, v/v) containing 0.1% formic acid. Melatonin content was expressed as ng/g dry weight (DW).

Phenolic Compound Extraction and Determination. Extraction of phenolic compounds was carried out following the method of Gou et al.[20] with some modifications. Raw and germinated flours (2 g) were blended using 30 mL of 80% chilled acetone (1:2, w/v) for 5 min and vortex for 10 min. The homogenates were then centrifuged at 2000*g* for 5 min. Supernatants were collected, and the extraction was repeated three times. All the supernatants were pooled and evaporated until 10% of the supernatants remained. The soluble free phytochemical extracts were brought to 10 mL in water and were kept at −40 °C until analysis. For the extractions of bound phytochemicals, the residues from the above soluble free extraction were flushed with N_2 and hydrolyzed directly with 20 mL of 4 M NaOH at room temperature for 1 h with shaking. The mixture was acidified to pH 2 with concentrated HCl, centrifuged at 3000*g* for 5 min, and extracted three times with ethyl acetate. The ethyl acetate fractions were evaporated to dryness and were reconstituted into 1.5 mL of methanol and stored at −40 °C until analysis.

Phenolic compounds were determined by Folin−Ciocalteu colorimetric method according to Singleton et al.[36] using gallic acid as standard. Phenolic compounds were expressed as mg GAE/100 g DW.

Oxygen Radical Absorbing Capacity (ORAC) Assay. The above methanol extracts were used for determining the radical scavenging activity by the ORAC method using fluorescein as a fluorescence probe.[37] Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4), and the final assay mixture (200 μL) contained fluorescein (70 nM), 2,2-azobis(2-methyl-propionamidine)-dihydrochloride (12 mM), and antioxidant standard (Trolox [10−80 μM] or sample extracts). A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) was used to read fluorescence at wavelengths of 485 nm excitation and 520 nm emission. The equipment was controlled by the Fluostar Galaxy software version (4.31-0) for fluorescence measurement. Black 96-well untreated microplates (PS Balck, Porvair, Leatherhead, UK) were used. The plate was automatically shaken before preincubation, and the fluorescence was recorded every minute for 80 min. All reaction

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mixtures were prepared in duplicate, and at least three independent runs were performed for each sample. Fluorescence measurements were normalized to the oxidation control (phosphate buffer) and stability control (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \text{AUC antioxidant} - \text{AUC blank}$$

The net AUC was plotted against the antioxidant concentration, and the regression equation of the curve was calculated. The ORAC value was obtained by dividing the slope of the latter curve by the slopes of the Trolox curve obtained in the same assay. Final ORAC values were expressed as μmol of Trolox equivalents/g of dry seed (μmol TE/g DW).

Sensory Analysis. Two different hummuses were prepared using lentil sprouts germinated for 10 days with and without (control) melatonin in the watering. Sensory evaluation was carried out in two sessions involving in total 40 trained and 60 untrained panelists. Sensory analysis was performed using three different tests; a descriptive test and two affective tests. To assess the sensory profile, each evaluated character was scored in a 10-point intensity scale (1 (lowest) to 10 (highest)). Color, odor, fluidity, consistency, texture, astringency, aftertaste, and flavor of the selected hummus, A (hummus prepared with enriched melatonin lentil sprouts) and B (hummus prepared without enriched melatonin lentil sprouts), were tested by 40 trained judges. For each attribute, the average of the panelists' scores was calculated. Satisfaction tests consisted of a 9-point verbal scale showing the acceptance of the product (like extremely = +4; like very much = +3; like moderately = +2; like slightly = +1; neither like nor dislike = 0; dislike slightly = −1; dislike moderately = −2; dislike very much = −3; dislike extremely = −4). Hummus was considered acceptable if their mean value for overall quality scores was equal to or above 0 (neither like nor dislike). Sensory evaluation was completed by a paired preference test. Untrained judges should choose between both samples tested, in order to assessing the distribution of panelists' preferences.

Statistical Analysis. Each sample was analyzed in triplicate. Data were expressed as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance (ANOVA) and post hoc Duncan's test. Student's t test (independent samples, 2 groups) was used to analyze differences in satisfaction between both hummus samples. Differences in the preference between these hummuses were studied by a binomial test and were considered to be significant at $p < 0.05$. The statistical analysis was performed using SPSS 21.0.

RESULTS AND DISCUSSION

Melatonin. *Melatonin Content in Whole Lentil and Bean Sprouts.* Raw kidney beans exhibited higher melatonin content (1.0 ng/g DW) than lentils (0.5 ng/g DW). These contents can be considered as natural sources of melatonin if they are compared with other plant foods; in general, seeds, such as almond, sunflower, and mustard, exhibited higher melatonin content.[9,38−40] Earlier studies indicated that traditional germination increased melatonin levels of different seeds.[31−33] However, these contents might not be sufficient to be incorporated as dietary supplements to increase melatonin in blood plasma in humans and, consequently, their scavenging and antioxidant actions. Thus, adding exogenous melatonin during watering might be a strategy to increase their melatonin levels during germination processing.

Germination with exogenous melatonin watering (20 μM) showed significant increases of melatonin in both legumes (Figure 2). In lentil sprouts, melatonin concentration increased from 0.5 ng/g DW in raw seeds to 1089.8 ng/g DW at the seventh day. However, a sharp melatonin decrease (42% with respect to the seventh germination day) was observed, reaching to 630.4 ng/g DW on the tenth day. Kidney bean sprouts presented lower melatonin contents than lentil sprouts throughout the whole germination process, although initially raw kidney beans exhibited higher melatonin levels than lentils. At the sixth day of germination, melatonin reached the highest levels in kidney bean sprout (529.1 ng/g DW). Thereafter, its content decreased gradually to 124.9 ng/g DW (76% decrease with respect to sixth germination day).

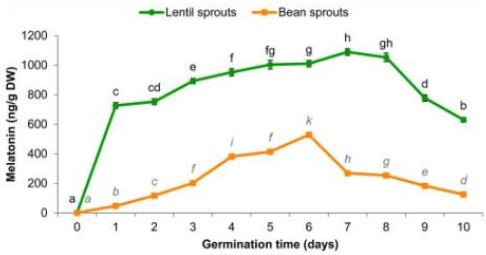

Figure 2. Melatonin contents in lentil (a) and kidney bean (b) sprouts grown with 20 μM melatonin in watering. Points with different letters significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

From these results, both lentil and kidney bean sprouts experienced a significant decrease of melatonin from the sixth or seventh germination day depending on the type of legumes; these reductions may be interpreted through melatonin metabolism.[33] The process of adding exogenous melatonin to the water increased the melatonin levels in these legumes, in contrast to those germinated by the traditional method (2.5 and 9.4 ng/g DW in lentil and kidney bean, respectively).[32]

It is worth emphasizing that sprouts germinated with exogenous melatonin watering absorbed up to 24% of added melatonin in the case of lentils and up to 11% in kidney beans. These differences were likely due to seed physiological factors such as thickness and surface area of the seed coats. Lentils exhibit seed coat thickness between 25 and 35 μm, depending on cultivar, being 8% of whole seed. However, kidney beans are associated with relatively thicker seed coats (40−60 μm), representing 12% of seeds.[41] Therefore, kidney bean seed coats might not allow melatonin diffusion as well as lentil ones.

Melatonin Content in Lentil Sprout Tissues. There is scarce information about melatonin content in sprouts after using an enriched watering. Because lentil exhibited higher melatonin levels than kidney bean sprouts, a detailed study was designed to measure this bioactive compound in the different tissues of lentil sprouts (seed coat, endosperm, and radicle). From the third day of germination, melatonin levels were higher in the seed coats than in the other tissues (endosperm and radicle) (Figure 3), increasing in all sprout tissues up to the eighth germination day. Afterward, melatonin decreased sharply (34%) in the seed coat from 897.2 ng/g DW to 589.6 ng/g DW, reaching lower levels than those found on the third day. In case of endosperm and radicle, they exhibited lower melatonin levels (92.1 ng/g DW and 26.4 ng/g DW, respectively), but significant increases were observed at the end of germination

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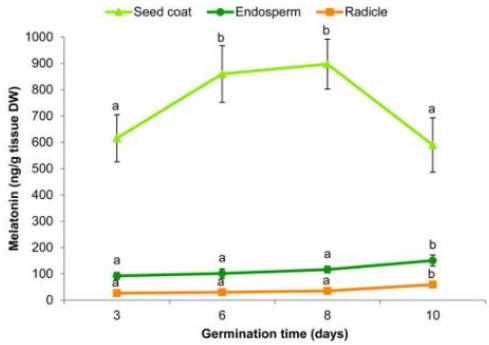

Figure 3. Melatonin contents in the different tissues (seed coat, endosperm, and radicle) of lentil sprout grown with 20 μM melatonin in watering. Points with different letters significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

period (tenth germination day), showing 150.3 ng/g DW and 58.9 ng/g DW in endosperm and radicle, respectively.

Melatonin Effects on Radicle Development. Melatonin exhibits auxin-like activity, promoting vegetative plant growth in a similar way to indole-3-acetic acid (IAA).[12] Indeed, significant enhancement ($p < 0.05$) of development of roots was observed as in lentil and in kidney bean sprouts (Figure 4).

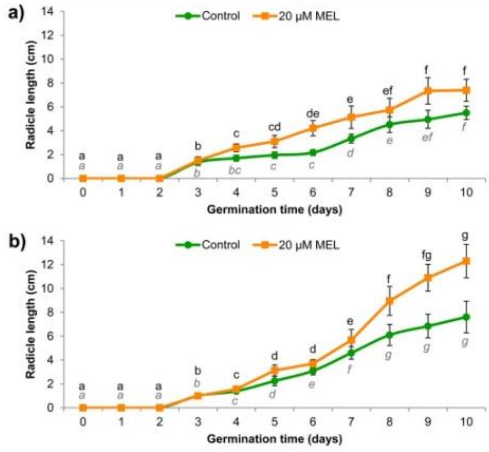

Figure 4. Radicle development in lentil (a) and kidney bean (b) sprouts grown with 20 μM melatonin in watering. Points with different letters significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

The radicles of lentils watered with 20 μM melatonin grew 1.4-fold more than those watered with H_2O (control); in the case of kidney beans, the increase was 1.6-fold. As described in the literature, the benefits of melatonin in plants vary with concentration. Germination rate and root development can be enhanced or inhibited.[12,15,17,42,43] From our results, 20 μM melatonin solution may suggest use as a correct concentration as biostimulator, as reported Janas and Posmyk.[16] Melatonin is likely to increase the final germination percentage besides the seed growth; therefore, sprout production becomes more economical and may increase sales and mark-up.

Furthermore, melatonin content in endosperms and radicles show positive correlations with the length of the sprouts (r = 0.877−0.879, $p < 0.01$) (Table 1). The increase of melatonin content could be explained not only by the growth stimulation of added melatonin but also by the influence of the germination.

Phenolic Compounds. The free (FPC), bound (cell wall-associated; BPC), and total phenolic compounds (TPC) of lentil and kidney bean sprouts are shown in Figure 5. Raw lentils and kidney beans exhibited lower TPC (487.5 mg GAE/100 g DW and 379.1 mg GAE/100 g DW, respectively) in comparison to TPC found in broad and red beans or in black beans by Amarowicz et al.[44] and Guajardo-Flores et al.[45] Nevertheless, TPC were higher compared with those found in mung bean by Guo et al.[20] and by Huang et al.[46] FPC and BPC in raw legumes were similar to those reported by Aguilera et al.,[32] FPC being the main fraction (95−98%).

Germination with exogenous melatonin watering induced changes in phenolic compound concentrations. Lentils showed slight decreases in a time-dependent manner in FPC from the first day (92% of TPC) to the tenth day (89% of TPC), and inversely, BPC increased 17%, reaching 44.3 mg GAE/100 g DW, attributable to the glycosylation of FPC. The highest decrease in TPC was found on the sixth day (21% of reduction from raw seed), and no more significant decreases were shown. On the tenth day of germination, lentil sprouts displayed 393.7 mg GAE/100 g DW (81% of the initial TPC content). Kidney beans exhibited no significant decrease in TPC nor in FPC. Nonetheless, a slight increase in BPC was shown from the eighth day. Kidney bean sprouts exhibited 36.5 mg GAE/100 g DW, 57% higher BPC compared with raw kidney beans.

Several authors have previously reported decreases in the phenolic content of legumes following germination.[47−49] A similar trend was found by Aguilera et al.,[32] where decreases in TPC in lentils were more accentuated after a traditional germination procedure. The activation of endogenous enzymes during germination could bring about changes in the phenolic composition of beans and lentils.[50] Thus, decreases in TPC could be attributed to the increases in activity of polyphenol oxidase and other catabolic enzymes, as observed by Khandelwal et al.[48] Furthermore, it has been reported that during germination procyanidins and catechins could condense to give more highly polymerized forms.[51] Oxidation could also be the cause of TPC decrease.[47] Melatonin, as antioxidant, could protect the sprout against oxidation, avoiding phenolic compound degradation. The results obtained compared with those obtained by Aguilera et al.[32] could corroborate the above hypothesis.

Antioxidant Activity. Both legumes revealed significant increases in the antioxidant capacity along the germination process (Figure 6), although lentils exhibited higher antioxidant capacity than beans. The antioxidant capacity of lentils increased 1.3 times (85.3 μmol TE/g tissue DW) during the germination process and the capacity of beans increased 1.7 times (55.8 μmol TE/g tissue DW). It is noticed that when melatonin content decreased during germination, the antioxidant capacity levels of these sprouts increased. This fact could be attributed to higher accumulation of compounds as melatonin metabolites from its catabolism. These compounds (AFMK, AMK, and cyclic 3-hydroxymelatonin) are more highly

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Table 1. Correlation Coefficients between the Contents of Melatonin, Phenolic Compounds, and Antioxidant Capacity in Lentil and Bean Sprouts[a]

Correlations in Lentil Sprouts												
	MEL	MEL-SC	MEL-END	MEL-RDC	length	TPC	FPC	BPC	AC	AC-SC	AC-END	AC-RDC
MEL	1	0.792[c]	−0.572	−0.730[c]	0.497	0.349	0.199	−0.379	0.855[c]	0.129	−0.262	−0.041
MEL-SC		1	0.024	−0.182	0.096	0.503	0.476	0.497	0.204	−0.021	0.371	0.550
MEL-END			1	0.970[c]	0.877[c]	0.263	0.704[b]	0.217	0.849[c]	−0.198	0.902[c]	0.788[c]
MEL-RDC				1	0.879[c]	0.105	0.555	0.053	0.820[c]	−0.264	0.811[c]	0.649[b]
length					1	0.328	0.576[b]	−0.398	0.827[c]	−0.590[b]	0.820[c]	0.661[b]
TPC						1	0.390	0.994[c]	−0.441	0.538	0.385	0.506
FPC							1	0.320	0.353	−0.185	0.704[b]	0.698[b]
BPC								1	−0.490	0.625[b]	0.349	0.498
AC									1	−0.452	0.855[c]	0.721[c]
AC-SC										1	−0.157	−0.001
AC-END											1	0.900[c]
AC-RDC												1

Correlations in Kidney Bean Sprouts						
	MEL	length	TPC	FPC	BPC	AC
MEL	1	0.051	−0.257	0.237	−0.301	0.338
length		1	−0.130	0.789[c]	−0.249	0.942[c]
TPC			1	0.286	0.982[c]	−0.151
FPC				1	0.139	0.838[c]
BPC					1	−0.275
AC						1

[a]MEL, melatonin; SC, seed Coat; RDC, radicle; END, endosperm; TPC, total phenolic compounds; FPC, free phenolic compounds; BPC, bound phenolic compounds; AC, antioxidant capacity. [b]Significant correlation for $p < 0.05$. [c]Significant correlation for $p < 0.01$.

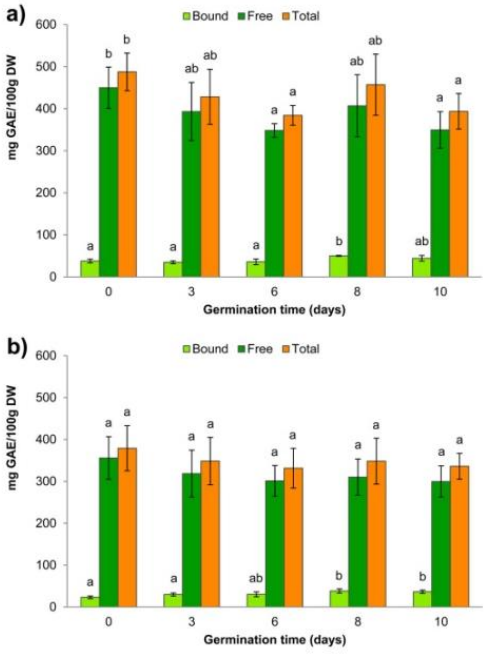

Figure 5. Phenolic compound contents in lentil (a) and kidney bean (b) sprouts grown with 20 μM melatonin in watering. Bars with different letters significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

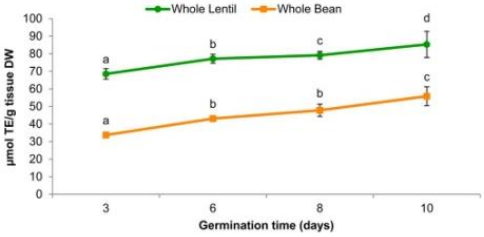

Figure 6. Antioxidant capacity (μmol TE/g DW) in lentil and kidney bean sprouts grown with 20 μM melatonin in watering. Points with different letters significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

effective scavengers than melatonin.[5,16,39,52] Melatonin showed a positive correlation with antioxidant capacity in lentil sprouts ($r = 0.855$, $p < 0.01$), but with bean sprouts the correlation was not significant (Table 1).

Because melatonin and phenolic compounds present relevant antioxidant properties, the study of antioxidant capacity was carried out in different tissues of the lentil sprouts; some of them had shown significant levels of these bioactive components (Table 2). Thus, seed coats were the tissues with the highest antioxidant capacity, 6-fold higher compared with endosperms, and they exhibited a significant decrease along germination. As was expected, endosperms showed the lowest antioxidant capacity during germination, although their levels increased to 24% on the tenth day of this process. Finally, radicles exhibited 3-fold higher antioxidant capacity than endosperms but did not show a significant enhancement during germination. Endosperm and radicle exhibited different behaviors compared with that reported by Guajardo-Flores et

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Table 2. Antioxidant Activity (μmol TE/g DW) in Different Tissues (Seed Coat, Endosperm, and Radicle) Of Lentil Sprouts Germinated with 20 μM Melatonin in Watering

germination time (days)	lentil sprouts		
	seed coat	endosperm	radicle
3	278.3 ± 25.4 b	44.6 ± 5.6 a	135.4 ± 9.7 a
6	234.8 ± 14.6 a	50.4 ± 4.3 ab	154.5 ± 20.4 a
8	228.5 ± 14.2 a	51.2 ± 3.3 ab	155.9 ± 20.6 a
10	225.9 ± 18.5 a	55.2 ± 3.7 b	161.3 ± 21.3 a

Results are reported as mean ± SD (n = 3). Mean values of each tissue of the lentil sprout followed by different letters significantly differ when subjected to Duncan's multiple range test (p < 0.05).

al.;[45] these differences could be attributed to aspects related to the germination process and watering with melatonin.

Thus, melatonin concentration reveals strong correlations with antioxidant capacity in lentil endosperms and whole sprouts (r = 0.902 and r = 0.855, respectively, p < 0.01). Analogous correlation is established between melatonin and their antioxidant capacity in the radicles. The length of the radicles exhibits positive correlation with the antioxidant capacity in lentils (r= 0.827, p < 0.01) and in beans (r = 0.942, p < 0.01), showing the positive effect of the germination process with enriched melatonin watering on the final antioxidant status of the sprouts.

Sensory Analysis of the Hummus. Figure 7 shows a spider-web diagram comparing descriptive profile results. Most

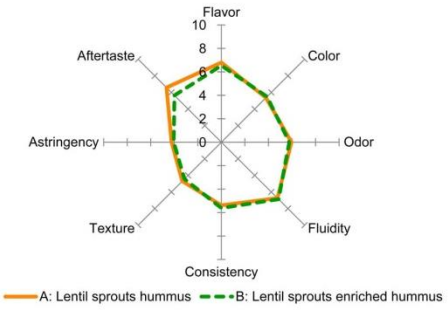

Figure 7. Spider-web diagram of sensorial attributes in lentil hummus. Mean scores (n = 40).

attributes such as flavor, color, aroma, fluidity, and consistency present similar scores, equal to or higher than five. Texture and astringency exhibit a score lower than five, but similar between A (nonenriched hummus) and B (enriched hummus). Only aftertaste brought about different points (7 in case of hummus A and 6 in hummus B). Therefore, melatonin enriched watering seems not to have any impact on the final product sensory quality, although radicle development is different in lentil sprouts germinated with enriched or nonenriched watering, which could increase green taste and grassy odor. Germination could have enhanced bitter taste and aftertaste, reducing sweet flavor and also showing more fibrousness.[40,42,49]

Satisfaction test results (n = 60) revealed a great acceptance of these hummuses; mean scores for the two samples exceeded the acceptance level (A = 1.1 and B = 1.3); t-test showed no significant differences between samples (p = 0.46). Regarding the preference test (n = 60), conclusions were reaffirmed; results do not show statistical significant difference (p = 0.37) (26 individuals preferred hummus A against 34 who preferred B). The panel found the lentil sprout hummus as flavorful products and indicate their probable product purchase if they could be found in the market. Thus, the melatonin-enriched hummus might be a good alternative to increase blood melatonin levels and, thereby, equilibrate the oxidative balance of the organism, as well as promote health, by increasing the dietary intake of plant-based foods.

This alternative method of germination with 20 μM melatonin in watering might be valid in sprout production, either lentils or beans. Adding exogenous melatonin into legume germination seems to increase its production and sprout healthiness, providing higher food quality. Lentil sprouts contained more melatonin than bean sprouts; but their concentration was considerably higher than other plant foods. Both sprouts showed good characteristics in order to be added to healthy diet. Therefore, these sprouts could be employed as functional ingredients by food industry, when developing melatonin-enriched novel foods. They might have an important nutritional impact because of melatonin's beneficial effects for human health.

AUTHOR INFORMATION

Corresponding Author

*E-mail: maria.martin@uam.es. Tel: 0034 91 497 8678. Fax: 0034 91 497 3826.

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ABBREVIATIONS USED

AFMK, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine; AMK, N(1)-acetyl-5-methoxykynuramine; BPC, bound phenolic compounds; DW, dry weight; FPC, free phenolic compounds; GAE, gallic acid equivalents; HPLC-MS/MS, high-performance liquid chromatography tandem mass spectrometry; MEL, melatonin; MRM, multiple reaction monitoring; TE, Trolox equivalents; TPC, total phenolic compounds; WW, wet weight

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Publicación V. El consumo de germinados de judía influye sobre la melatonina y capacidad antioxidante como biomarcadores de niveles en ratas

Yolanda Aguilera, Miguel Rebollo-Hernanz, Teresa Herrera, L. Tábata Cayuelas, Pilar Rodríguez-Rodríguez, Ángel L. López de Pablo, Silvia M. Arribas, María A. Martín-Cabrejas. Intake of bean sprouts influences melatonin and antioxidant capacity biomarkers levels in rats. *Food Funct*. **2016**, Mar 16; 7(3): 1438-45.

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RESUMEN

La melatonina es una hormona antioxidante endógena, que se reduce a lo largo de la vida, asociándose bajos niveles con algunas enfermedades crónicas. La germinación de las leguminosas incrementa los niveles de melatonina en plantas, obteniéndose germinados como una fuente alimentaria de esta hormona. Sin embargo, la información sobre su biodisponibilidad después de su consumo es insuficiente. El objetivo de este estudio fue evaluar el efecto en ratas de la ingesta de germinados de judía en los niveles plasmáticos, así como la concentración de metabolitos secundarios derivados de la melatonina (6-sulfoximelatonina), compuestos fenólicos totales y capacidad antioxidante total. Además, se comparó la biodisponibilidad del plasma derivada de germinados de judía frente al consumo melatonina sintética. Se les administraron extractos acuosos de judías germinadas (6 días). Se utilizaron ratas macho jóvenes Sprague Dawley, obteniéndose muestras de sangre y orina antes y después de 90 min de la administración de extracto de germinado de judía a través de una sonda nasogástrica. Los niveles plasmáticos de melatonina incrementaron después de la ingestión (16%, $p < 0,05$). Este incremento esta correlacionado con el contenido en orina de 6-sulfatoximelatonina, el principal biomarcador de niveles plasmáticos de melatonina ($p < 0,01$). Sin embargo, los niveles de compuestos fenólicos y capacidad antioxidante no mostraron ninguna variación significativa. La comparación de biodisponibilidad entre el contenido de melatonina en germinados de judía y la solución sintética evidenciaron una ligera elevación de niveles de melatonina plasmáticos (17%) en ratas alimentadas con la solución sintética de melatonina. Se concluye que los germinados de judía podrían ser una buena fuente de melatonina y otros compuestos bioactivos en la dieta, debido a los efectos beneficiosos que tienen sobre la salud.

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Intake of bean sprouts influences melatonin and antioxidant capacity biomarker levels in rats

Yolanda Aguilera,†[a] Miguel Rebollo-Hernanz,†[a] Teresa Herrera,[a] L. Tábata Cayuelas,[a] Pilar Rodríguez-Rodríguez,[b] Ángel L. López de Pablo,[b] Silvia M. Arribas[b] and María A. Martin-Cabrejas*[a]

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Melatonin is an endogenous antioxidant hormone, which reduces with ageing and the low levels are associated with some chronic diseases. Germination of legumes increases the plant levels of melatonin, making sprouts a suitable food source of this hormone. However, information on its bioavailability after consumption is lacking. We aimed to evaluate in rats the effect of kidney bean sprout intake on the plasma levels of melatonin and metabolically related compounds (serotonin, 6-sulfatoxymelatonin), total phenolic compounds and total antioxidant capacity. In addition, we compared the plasma bioavailability derived from kidney bean sprouts *versus* synthetic melatonin intake. Kidney beans were germinated for 6 days and an extract was prepared in water. Male young Sprague Dawley rats were used; blood and urine samples were obtained before and after 90 min of administration of kidney bean sprout extract *via* a gavage. The plasmatic melatonin levels increased after sprout ingestion (16%, $p < 0.05$). This increment correlated with the urinary 6-sulfatoxymelatonin content, the principal biomarker of plasmatic melatonin levels ($p < 0.01$). Nevertheless, the phenolic compounds and antioxidant capacity levels did not exhibit any significant variation. The comparison of the bioavailability between the melatonin contained in the kidney bean sprouts and in a synthetic solution evidenced slightly higher levels of plasmatic melatonin (17%) in rats fed with the solution of synthetic melatonin. We conclude that kidney bean sprouts could be a good source of dietary melatonin and other bioactive compounds known to have health benefits.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a molecule with a wide range of cellular and physiological actions.[1] Melatonin shows potent antioxidative properties as a direct free radical scavenger[2] as well as through its catabolites.[3,4] Moreover, it has the ability to stimulate endogenous antioxidant enzymes (*e.g.* catalase, superoxide dismutase, *etc.*).[5] An increase in plasmatic melatonin levels has been correlated with reduced oxidative stress.[6] Exogenous melatonin is widely used for therapeutic purposes. Up to now, the use of melatonin has been restricted to the improvement of sleep quality, the alleviation of subjective feelings of jet lag, and the reduction of sleep onset latency.[7] However, numerous studies concluded that melatonin could also be associated with the prevention of different diseases related to ageing and oxidative stress, including type 2 diabetes, cardiovascular diseases, neurodegenerative disorders or cancer.[8–11]

Most generated knowledge about melatonin beneficial effects has been gained with exogenous synthetic melatonin either *in vitro* or *in vivo* (experimental animals and humans) and much less is known about the effect of diet on the synthesis and plasmatic levels of melatonin. Recent findings claim the importance of food intake on the plasmatic level of melatonin.[12] It has been detected that fasting periods and energy restriction decrease the nocturnal secretion of melatonin;[13] high-calorie food also modifies melatonin secretion.[14] In addition to the relevant influence of the light-dark cycle, diet and nutrients might also modulate the melatonin plasmatic levels.[14] It has been proposed that the consumption of plant foods containing melatonin may improve human health due to its biologic activities and bioavailability.[15] For example, it has been reported that the ingestion of products rich in melatonin increases the plasmatic levels of the hormone or the excreted urinary metabolite 6-sulfatoxymelatonin (aMT6s).[6,16] Nevertheless, more studies are needed to explore the melatonin bioavailability and plasma fluctuation levels after intake of foods rich in this compound.

[a]*Instituto de Investigación de Ciencias de la Alimentación (CIAL), Facultad de Ciencias, Universidad Autónoma de Madrid, Spain. E-mail: maria.martin@uam.es; Tel: +34 91 001 7913; Fax: +34 91 497 3826*

[b]*Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Spain*

† Both authors contributed equally to this work and should be considered as the first authors.

The presence of melatonin in different legumes has recently been studied.[17–19] These foods are important sources of proteins, vitamins and minerals. They have recently received further attention because of their health benefits on chronic disease prevention, attributed to their relevant soluble and insoluble fibers, slow digestive starch, prebiotic oligosaccharides and phenolic content.[20,21] Some of these compounds possess antioxidant properties that are correlated with their potential health benefits in aging and prevention of oxidative stress-associated diseases.[21]

Legume germination is a simple and commonly used process for improving their nutritional value in many countries. This processing significantly reduces the non-nutritive components, increases the digestibility of proteins and bioavailability of certain minerals and vitamins. Furthermore, we have previously demonstrated that germination enhances the content of antioxidant bioactive compounds, including melatonin.[19,22] Hence, the consumption of germinated legumes could be a strategy to prevent, through the diet, the mentioned diseases associated with oxidative stress.

Thus, this study aims to determine in rats whether the intake of germinated kidney beans (*Phaseolus vulgaris* L.) would alter the melatonin levels and antioxidant capacity in the plasma, as well as potentially related biomarkers such as serotonin and total phenolic compounds in the plasma, and aMT6s in urine. In addition, it also assessed the food matrix effect on melatonin absorption, comparing its levels and antioxidant capacity after synthetic melatonin or kidney bean sprout extract consumption.

Materials and methods

Kidney bean sprout extracts

Kidney beans (*Phaseolus vulgaris* L. var. *Pinta*), provided by Institute of Food Science, Technology and Nutrition (CSIC, Madrid), were germinated according to Aguilera *et al.*[23] This process showed good viability, 98% being the percentage of germination. Sprouts were freeze-dried, milled, packed in vacuum bags, and stored at −20 °C. Analyses of sprouts in triplicate were carried out to determine the melatonin, phenolic compounds, and antioxidant capacity as described previously.[19]

The extract from kidney bean sprouts was prepared as follows: kidney bean sprout flour (20 g) was mixed with ethanol (150 mL) and shaken for 16 h at 4 °C in the dark. The mixture was sonicated for 15 min and filtered under vacuum through 11 μm filters (Whatman). The extract was evaporated at 30 °C to dryness and redissolved in 3 mL PBS buffer. The melatonin content in the extract was analyzed, being 10.6 μg. The extract was dissolved in 3 mL Milli-Q water. A synthetic melatonin (≥98%; Sigma-Aldrich Química, Spain) solution of the same concentration as the sprout extract was prepared in Milli-Q water, shaken, and sonicated for 15 min, at the same level as the kidney bean extract.

Animals and experimental design

Experiments were performed in Sprague Dawley rats from the colony maintained at the Animal House facility of the Universidad Autónoma de Madrid (Fig. 1). All experimental procedures were approved by the Ethics Review Board of Universidad Autónoma de Madrid and conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised in 1996), the Spanish legislation (RD 1201/2005) and the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Experiment 1. The rats were housed under controlled conditions of 22 °C, 40% relative humidity and 12/12 light/dark photoperiods. After weaning (day 21), the rats were kept for 23 days under the changed light/dark cycle. They were fed *ad libitum* with a breeding diet (SAFE A03) containing 51.7% carbohydrates, 21.4% protein, 5.1% lipids, 3.9% fiber, 5.7% minerals and 12.2% humidity (Safe Augy, France). Drinking water was filtered by UV, mechanical and chemical treatments, and provided *ad libitum* in all cases. All the animals were housed in buckets of 36.5/21.5/18.5 cm (length/width/height) on aspen wood bedding, which was replaced once a week. The animal health monitoring indicated that they were free of any

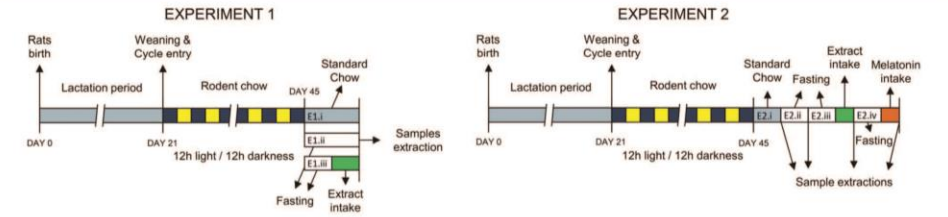

Fig. 1 Illustrative experimental design of the experiments carried out. In Experiment 1, at the age of 45 days the rats were subjected to different feeding conditions: (E1.i) standard rat chow without extract (control), (E1.ii) 24 h-fasting period (fasting), or (E1.iii) kidney bean extract administration after 24-h fasting, followed in all the cases by blood and urine sampling. In Experiment 2, samples were obtained from the same rat group; (E2.i) after common feeding (control), (E2.ii) after 12 h-fasting (fasting), (E2.iii) after 12 h-fasting and the intake of the bean extract (KB extract), and (E2.iv) after the intake of a melatonin solution (MEL).

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pathogens that may interact with any of the parameters studied. The health and welfare of the animals was monitored by the staff at least once a day. At the end of this period (days 44–45), the rats were subjected to three different feeding conditions (n = 8 per group): (E1.i) standard rat chow without extract (control), (E1.ii) a 24 h-fasting period without extract (fasting), and (E1.iii) a 24 h fasting period followed by administration of the KB extract (3 mL containing 10.6 μg of melatonin). Administration was performed through a gavage using a suitable intubation cannula and was carried out by a specialised Animal House technician (Table 1).

Thereafter, the rats were individually caged and a cling film was placed below the cage to obtain the urine samples. After 90 min of extract administration, all rat groups were anesthetized by CO_2. Urine was collected from the cling film with a pipette and transferred to a vial. The blood was collected by cardiac puncture, transferred to vials containing 5% heparin and centrifuged at 4 °C for 15 min at 2100g. The plasma was then divided into aliquots in 1 mL vials and kept frozen at −80 °C to assess several biomarkers related to melatonin metabolism. All experiments were carried out at 10:30 a.m., which was the peak time of melatonin production under the changed light/dark cycle.

Experiment 2. A second group of rats was used to test, in paired experiments, the differences in plasma melatonin with different nutritional interventions: (E2.i) rat chow, (E2.ii) 12 h-fasting, (E2.iii) KB extract after 12 h-fasting and (E2.iv) pure melatonin 12 h-after fasting (male rats (n = 8)). For these experiments, after the nutritional intervention, a blood extraction was obtained at 90 min, through sublingual bleeding. The different interventions and blood sampling were carried out with an interval of six days.

Biochemical determinations

Melatonin. Plasma was evaporated to dryness by using an evaporator centrifuge (SpeedVac SC 200, Savant, USA). The residues were dissolved in distilled water and melatonin levels were determined by using a competitive enzyme immunoassay kit (Melatonin ELISA, IBL-International, Hamburg, Germany) according to the manufacturer's instructions. The kit is characterized by an analytical sensitivity of 1.6 pg mL^{-1} and high analytical specificity (low cross-reactivity).

6-Sulfatoxymelatonin (aMT6s). Urine was diluted in Tris-buffered saline (TBS) and protected from direct sun light. aMT6s levels were determined by using a competitive enzyme immunoassay kit (Melatonin ELISA, IBL-International, Hamburg, Germany) according to the manufacturer's instructions. The assay sensitivity was 1.0 ng mL^{-1}.

Serotonin. Plasma was evaporated to dryness by using an evaporator centrifuge (SpeedVac SC 200, Savant, USA). The residues were dissolved in distilled water and melatonin levels were determined by using a competitive enzyme immunoassay kit (Melatonin ELISA, IBL-International, Hamburg, Germany) according to the manufacturer's instructions. The kit is characterized by an analytical sensitivity of 2.68 ng mL^{-1} and high analytical specificity (low cross-reactivity).

Total phenolic compounds (TPC). Phenolic compounds were determined by the Folin–Ciocalteu colorimetric method according to Singleton *et al.*[24] using gallic acid as the standard. Phenolic compounds were expressed as mg GAE mL^{-1}. In a tube, 3 mL Milli-Q water, the 50 μL sample and 250 μL Folin–Ciocalteu reactive were merged. After 3 min of repose, 750 μL of Na_2CO_3 (20%) and 950 μL of Milli-Q water were added, mixed and placed in the dark for 120 min. Absorbance was measured at 760 nm.

Antioxidant capacity

ORAC (oxygen radical absorbance capacity). The above plasma samples were used for determining the radical scavenging activity by the ORAC method using fluorescein as a fluorescence probe.[25] Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained fluorescein (70 nM), 2,2-azobis(2-methyl-propionamidine)-dihydrochloride (12 mM), and the antioxidant standard (Trolox or sample extracts). Fluorescence was read at 485 nm excitation and 520 nm emission. Black 96-well untreated microplates (PS Black, Porvair, Leatherhead, UK) were used. The plate was automatically shaken before pre-incubation, and the fluorescence was recorded every minute for 80 min. All reaction mixtures were prepared in duplicate and at least 3 independent runs were performed for each sample. Fluorescence measurements were normalized to the oxidation control (phosphate buffer) and stability control (no antioxidant). From the normalized curves, the area under

Table 1 Experimental conditions for Experiments 1 and 2 considering the intervention, rat population, weight, date of entrance and time into inverse photoperiod, sampling day and sampling time

	Experiment 1			Experiment 2			
	Control	Fasting	KB extract	Control	Fasting	KB extract	MEL
Intervention	None	24 hour-fast	24 hour-fast Bean extract	None	12 hour-fast	12 hour-fast Bean extract	12 hour-fast Melatonin
Rat population (N)	8 ♂	8 ♂	8 ♂	8 ♂			
Weight (g)	202 ± 12	193 ± 8	208 ± 10	201 ± 7			
Entrance into inverse photoperiod (day)	21	29	21	21			
Time into inverse photoperiod (days)	25	15	31	23	29	35	41
Sampling day	46	44	52	44	50	56	62
Sampling time	10:30	10:30	10:30	10:30	10:30	10:30	10:30

the fluorescence decay curve (AUC) was calculated as

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \text{AUC antioxidant} - \text{AUC blank}$$

The net AUC was plotted against the antioxidant concentration, and the regression equation of the curve was calculated. The ORAC value was obtained by dividing the slope of the latter curve between the slopes of the Trolox curve obtained in the same assay. The final ORAC values were expressed as mM Trolox equivalents (mM TE).

FRAP (ferric reducing ability of plasma). The FRAP assay was performed as previously described.[26] 1.5 mL of a working FRAP reagent [acetate buffer 0.3 M, pH 3.6, 10 mM tripyridyl *s*-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ (10 : 1 : 1) (v/v/v)] were warmed to 37 °C, and then 50 μL of plasma samples were added. The absorbance was recorded at 593 nm against the reagent blank after 10 min. FRAP values were calculated and expressed as μM Trolox equivalents (μM TE).

Statistical analysis

Each sample was analysed in triplicate. Data were expressed as mean ± standard deviation (SD). The data were analysed by one-way analysis of variance (ANOVA) and *post hoc* Duncan tests. The relationships between the analysed parameters were evaluated by computing the Pearson linear correlation coefficients setting the level of significance at $p < 0.05$ and $p < 0.001$. The statistical analysis was performed by using SPSS 21.0. Additionally, for curve-fitting analysis in ELISA assays, the results were processed by using the 4-parameter logistic nonlinear regression model, using OriginPro 8.5.

Results and discussion

Characterization of been sprout extract

It has been recently reported that germination has led to improvements in melatonin levels, bringing about significant increases of antioxidant activity in bean sprouts.[19,23] However, studies on the bioavailability of melatonin contained in these germinated legume seeds and the possible impact that their intake may have on health have not been performed.

Studies have demonstrated that the presence of melatonin in plants is universal and its levels vary widely.[27,28] Melatonin in legumes may be related to the protection of highly oxidizable lipids from oxidation, thereby preserving the seed viability for germination.[29] The performed germination was already evaluated in previous studies,[23] to maximize the load of these compounds and the antioxidant capacity. In the present study, melatonin was identified and quantified in germinated bean extracts with a value of 529.1 ng g^{-1} (Table 2). The extract also contained phenolic compounds (336.7 mg GAE per 100 g), lower than other bean varieties.[30,31]

Table 2 Kidney bean extract characterization including the melatonin content, total phenolic compounds (TPC), and antioxidant capacity measured by ORAC[a]

Melatonin (ng g^{-1})	TPC (mg GAE per 100 g)	ORAC (μmol TE per g)
529.1 ± 27.5	336.7 ± 35.8	43.1 ± 3.5

[a] Results are reported as mean ± SD (n = 3).

Both melatonin and phenolic compound levels, as well as other antioxidant phytochemicals, exhibit changes in their contents along the germination process.[19,32] The antioxidant capacity of the extract (43.1 μmol TE per g) was mainly due to the relevant content of phenolic compounds. The ORAC data were in agreement with the results reported in the literature for raw common bean varieties,[33] and other legumes, as lentils, chickpeas, or lupins.[32] Wu *et al.*[34] investigated ORAC in common foods and the results showed that kidney beans exhibited higher levels than other foods, including many fruits commonly believed to be rich in antioxidants. This extract was then used to feed one group of rats (KB extract).

Experiment 1. Fig. 2a shows the variations in the peak plasmatic melatonin levels in the control, 24-hour of fasting, and after 90 min of the administration of the kidney bean extract.

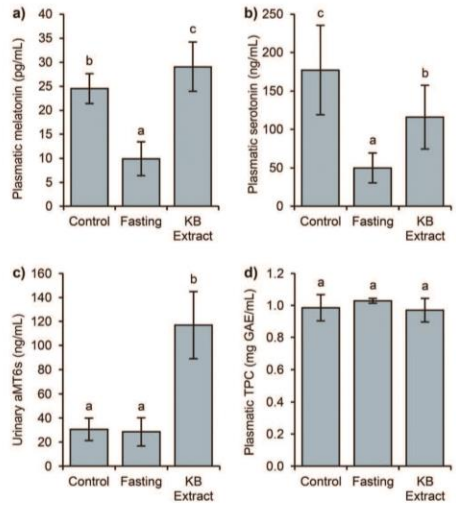

Fig. 2 Levels of melatonin (a), serotonin (b), aMT6s (c), and TPC (d) in the plasma and urine samples after the three different conditions (control, fasting, and KB extract) from Experiment 1. The results are reported as mean ± SD (n = 8).

The group of rats fed with *ad libitum* (control) showed 25 pg mL^{-1} of melatonin in the plasma while the rats subjected to fasting for 24 hours exhibited lower melatonin levels, reaching 10 pg mL^{-1}, 40% reduction with respect to the control. These results are in agreement with the data in humans[35,36] as well as in experimental animals,[49] suggesting an association between caloric restriction and lower melatonin plasmatic levels. However, there is still some controversy and the role of fasting in the fluctuation of melatonin levels is not completely understood.[13] The animals fasted for 24 h and given the extract (KB extract) showed 29 pg mL^{-1} plasma melatonin after 90 min from its administration, significantly higher levels (16%) than values obtained in the control group (control) and being 2.9-fold higher compared to the fasting group.

It is worth pointing out that in most studies the addition of exogenous melatonin to laboratory animals is mainly carried out in tap water.[37] Our results implied the possibility of using food as the melatonin source. Hence, a relevant content of melatonin contained in the diet may be absorbed by the gastrointestinal tract, increasing its level in the plasma. However, our results were not as high as melatonin levels reported after walnut ingestion.[6] Several factors might be involved in the plasmatic levels of melatonin; among them are the age of animals, the amount of ingested melatonin, intake period (from hours to weeks), *etc.*[6,38,39] In addition, the time of blood collection might have an influence.[40] It has been demonstrated that consumption of different fruits lead to variations in the plasmatic melatonin levels due to their different bioavailabilities.[39,41,42]

Furthermore, possible differences in the tryptophan content in plant matrices may also enhance the synthesis of extra-pineal melatonin by the biotransformation *via* serotonin to melatonin, for example in the gastrointestinal tract.[43]

Fig. 2b shows the serotonin variations in the above groups of rats. In the control group, the level reached 177 ng mL^{-1}. The 24-hour fasting group produced a drastic decrease of serotonin (72%), being 50 ng mL^{-1}. The group subjected to 24 h of fasting followed by the administration of germinated bean extract reached 116 ng mL^{-1} of serotonin, representing 1.3 fold higher than the fasting group. After 90 min of intake, an important increase of the serotonin levels was produced. Thus, the influence of diet seems relevant because after 90 min of its administration, the serotonin levels reached 65% of the level from the control group.

The levels of urinary 6-sulfatoxymelatonin (aMT6s), the major metabolite of melatonin in urine is considered to be a good indicator of melatonin in the plasma, showing a correlation with the plasmatic hormone levels.[41] The basal aMT6s level was 30 ng mL^{-1}, interestingly these levels remained after fasting (fast, 28 ng mL^{-1}), but a drastic increment of the aMT6s levels (4-fold) was detected after the intake of the bean extract (KB extract), compared to the basal and fasting levels (Fig. 2c). The KB extract results corroborated the previous studies which observed the association of vegetable and fruit intake with significant increases in the urinary aMT6s levels.[41,42] The aMT6s levels found after the bean extract intake brought about the high catalytic efficiency for melatonin sulfation in rats.[44] Melatonin is rapidly metabolized to 6-hydroxymelatonin which is further conjugated to aMT6s.[45] Likewise, the total intrinsic clearance rate of melatonin sulfation presents considerable species differences, being higher in rats than in humans or mice.[44] Consequently, the level of aMT6s increases rapidly after the intake of the bean extracts, rich in melatonin.

Regarding plasmatic phenolic compounds (Fig. 2d), all three studied groups exhibited statistically similar TPC levels (1 mg GAE mL^{-1}). In Sprague Dawley rats fed with tea, the achieved levels of TPC were similar to those found in the present work.[46] The plasmatic half-life of these compounds usually ranges from 2 to 8 h, but sometimes it can reach up to 12–24 h.[47] It has been shown that plasmatic phenolic levels generally exhibit sharp decreases one hour after the ingestion.[48,49] However, in our study, the levels of phenolic compounds in the plasma of 24 h-fasted rats did not show any decrease, probably due to their accumulation either in the plasma or in other tissues as some studies have previously demonstrated.[50] Their presence in the gut, principally in the large intestine, in higher quantities than in the plasma, seems to be the principal cause for the maintenance of the total phenolic load.[49] Because colonic microbiota mediates the formation of phenolic acids from larger phenolic compound polymers through glycoside hydrolysis, ring fission, and oxidation, the resulting metabolites can be absorbed and enter the systemic circulation.[49,51,52] Consequently, the TPC measurement after 24 h-fasting would show the content of all those compounds, coming from the diet of the rats, made mainly of plant foods (wheat, corn, wheat bran, barley, soybean, *etc.*). Likewise, as complex phenolic compounds and glycosides required to be transformed in the colon to be absorbed, in a short term intake of kidney bean extract, phenolic compounds might not reach this digestion stage, and not be bioavailable.

In this study, two assays (ORAC and FRAP) were selected to evaluate the antioxidant capacity because of the different antioxidant mechanisms assessed by these methods (ORAC by hydrogen atom transfer and FRAP by assessing single electron transfer). It is known that most antioxidants act by a combination of both mechanisms, and melatonin is no exception.[53] The ORAC data exhibited similar values, with no significant differences (Fig. 3).

In the same way, the FRAP antioxidant levels show no significant difference between the studied groups. Thus, the antioxidant capacity measured by the above assays may not reflect the total influence of melatonin in germinated bean extract on endogenous antioxidant capacity.

FRAP and ORAC assays only measure the free radical scavenging capacity and melatonin exhibits direct and indirect antioxidant actions,[54,55] including the stimulation of endogenous antioxidant enzyme expression.[5] Since we measured the antioxidant capacity after a single extract administration, we could not detect these indirect antioxidant effects of melatonin. A long term administration with kidney bean sprouts

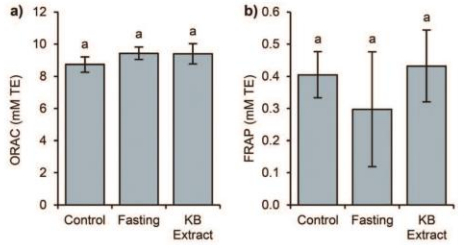

Fig. 3 Determinations of the levels of the antioxidant capacity in the plasma samples, measured by ORAC (a) and FRAP (b), after the three different conditions (control, fasting, and KB extract) of Experiment 1. The results are reported as mean ± SD (n = 8).

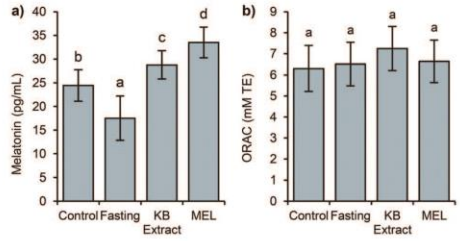

Fig. 4 Determinations of the levels of melatonin (a) and the levels the antioxidant capacity (ORAC) (b) in the plasma samples after the four different conditions (control, fasting, KB extract, and MEL) of Experiment 2. The results are reported as mean ± SD (n = 8).

could answer this question. In addition, it is possible that the results imply conservative estimation of antioxidant levels and cannot be just attributed to melatonin. As shown in other studies, the intake of food stuffs rich in melatonin such as cherries, grape juices or beers led to increases in the plasmatic antioxidant capacity. Nonetheless, these results could not be directly related to melatonin levels.[16,42,56]

Additionally, the Pearson linear correlation between the antioxidant capacity and the studied bioactive compounds was calculated, showing no relationship between them. Even if the statistics showed no correlation, we can assume that the antioxidant capacity measured by ORAC is mainly due to the levels of phenolic compounds, and other compounds not evaluated in this work. As it was mentioned, only the level of aMT6s in urine was correlated with the plasmatic melatonin level (r = 0.713, $p < 0.01$).

Experiment 2. According to our knowledge, there are no studies comparing the bioavailability of melatonin located within a plant food matrix with that of a synthetic melatonin solution. For this purpose, a fourth group was included (MEL), in this experiment. Hence, the same content of melatonin was given to both the KB extract and MEL groups (10.6 μg).

The melatonin levels displayed a similar behaviour as in the Experiment 1, corroborating the observed decrease of melatonin after a fasting period, and its sharp increase when the bean extract was consumed by the rats (Fig. 4).

Regarding the level of plasmatic melatonin in the fourth group (MEL), it showed a significantly higher level, compared to the rest of the groups. Therefore, the melatonin bioavailability in an aqueous solution resulted in 17% higher than that in the matrix of the kidney bean extract. From these results, it was highlighted that the food matrix influenced directly on the absorption of melatonin in the gut. Melatonin in the kidney bean extract is accompanied by other methanolic soluble compounds such as phenolics, which could modify its bioavailability, as described in other studies related to the evaluation of the dietary intake of melatonin from fruits.[41]

Concerning the antioxidant capacity, as observed in Experiment 1, the data remained similar in all groups, including the MEL group. Thus, the variations in the plasmatic levels of melatonin were not translated into differences in the antioxidant capacity.

Conclusions

Kidney bean sprouts have been demonstrated to contain great amounts of bioactive antioxidant compounds, especially phenolics, which constitute the main antioxidant phytochemicals found in the plasma. In addition, melatonin is available in the plasma after kidney bean sprout ingestion, which indicates that it is readily absorbed. The lack of increased plasma free radical scavenging capacity of kidney bean extract or pure melatonin is likely due to the masking effects of phenolic compounds. However, it is possible that increased plasma melatonin from food sources over prolonged time periods might exert similar indirect antioxidant actions, as previously described for pure compounds. In conclusion, germinated legumes are a suitable natural source of exogenous melatonin. However, additional work is still needed on this issue to determine the long term effects of dietary melatonin consumption on antioxidant defence systems and disease prevention. The health benefits derived from the dietary intake of melatonin are, until now, controversial, as it is not recognized if the chronic consumption of melatonin through the diet has physiological effects.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

aMT6s	6-Sulfatoxymelatonin
FRAP	Ferric reducing ability of plasma
GAE	Gallic acid equivalents
MEL	Melatonin

ORAC	Oxygen radical absorbance capacity
TE	Trolox equivalents
TPC	Total phenolic compounds

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Discusión General

IV. DISCUSIÓN GENERAL

La presente Memoria aporta información novedosa relativa a la presencia de compuestos bioactivos, destacando la presencia de la melatonina, a través del proceso de germinación en semillas de legumbres y hortalizas, y su capacidad antioxidante (*Publicaciones I, II, III y IV*). Asimismo, se realizó un estudio de la biodisponibilidad de melatonina en semillas de legumbres germinadas en animales de experimentación para evaluar e interpretar los posibles efectos tras la ingesta de un extracto rico en melatonina y su comparación con melatonina pura (*Publicación V*).

Los compuestos bioactivos o fitoquímicos son productos del metabolismo secundario de las plantas, de gran interés puesto que su consumo está asociado con efectos protectores de la salud. La germinación puede considerarse como una estrategia agronómica para mejorar la calidad nutricional de legumbres, debido al incremento de compuestos bioactivos.

Para desarrollar el **primer objetivo** propuesto en la presente Memoria, se ha evaluado la influencia del proceso de germinación durante un periodo de 4 días bajo condiciones lumínicas de 12h luz/12h oscuridad, en legumbres no convencionales (*Vigna unguiculata, Canavalia ensiformis, Mucuna pruriens y Lablab purpureus*) sobre los compuestos bioactivos más representativos, como son los compuestos fenólicos e inhibidores enzimáticos para incrementar la utilidad de estas semillas y su posible incorporación en la dieta (**Publicación I**). Se constató que las semillas presentan niveles de **compuestos fenólicos** muy diferentes entre ellas, lo cual podría ser debido al color que exhiben dichas semillas en su cubierta. Numerosos estudios indican que esta característica física se correlaciona positivamente con el contenido fenólico tal como se ha puesto de manifiesto en variedades de judías altamente pigmentadas (Xu y col., 2007). El proceso de germinación llevado a cabo en este estudio produce incrementos significativos de compuestos fenólicos ($p < 0,05$) que corroboran los mostrados por otros investigadores (Dueñas y col., 2009; Cevallos-Casals y Cisneros-Zevallos, 2010; Guo y col., 2012; Vernaza y col., 2012). Por lo tanto, la germinación modifica la composición fenólica cuantitativamente y ésta parece depender del tipo de leguminosa y de las condiciones del proceso.

Asimismo, también se analizaron las modificaciones producidas en el contenido de las familias estudiadas de compuestos fenólicos, catequinas y proantocianidinas en leguminosas no convencionales. Las catequinas se detectaron en todas las semillas así como en sus germinados, el contenido de estos compuestos se incrementó significativamente ($p < 0,05$) en todos los germinados estudiados, probablemente debido a la producción de metabolitos secundarios como los flavonoides (Bartolomé y

col., 1997; Dueñas y col., 2009). De igual manera, las proantocianidinas se han identificado en todas las semillas, llegando a alcanzar el 30% del contenido de compuestos fenólicos. Estos aumentos pueden ser debidos a los cambios bioquímicos producidos en la semilla durante la germinación como consecuencia de la condensación de polímeros (Ricardo-da-Silva y col., 1991). La relación proantocianidinas/catequinas es una aproximación relativa del grado de polimerización de las proantocianidinas. Este nivel resulta ser importante ya que desde el punto de vista nutricional, interesa que esta relación sea baja, ya que el número de interacciones de proantocianidina-proteína aumenta con el grado de polimerización (Ricardo-da-Silva y col., 1991). Los resultados obtenidos indican que el grado de polimerización es similar en todas las muestras a lo largo del proceso de germinación, produciéndose un descenso significativo. Este hecho se ha observado también en la germinación y fermentación de lentejas (Bartolomé y col., 1997).

En la caracterización nutricional de estas leguminosas no convencionales también se evaluó el contenido de **inhibidores enzimáticos** (α-amilasa e inhibidores de proteasas). Se conoce que la presencia de inhibidores, ampliamente distribuidos en legumbres, puede verse afectada tras el proceso de germinación. Los resultados obtenidos corroboran la ausencia de inhibidores de α-amilasa en todas las leguminosas no convencionales, tanto en las semillas como en sus germinados. En relación a los inhibidores de proteasas (tripsina y quimotripsina), su presencia fue detectada en todas las muestras, mostrando reducciones tras el proceso de germinación, siendo mayores los niveles de tripsina que quimotripsina en todas las leguminosas estudiadas. En general, la presencia de estos inhibidores disminuye durante este proceso, en legumbres tanto convencionales como no convencionales (Akpapunam y Sefa-Dedeh, 1997). Este hecho puede ser debido a las diferentes actividades de las proteasas endógenas, producido por el incremento de proteasas pre-existentes que son activadas (Vidal-Valverde y col., 2002).

En relación a la **capacidad antioxidante**, se realizó un estudio mediante las metodologías de FRAP y DPPH. La capacidad antioxidante inicial en estas semillas de legumbres no convencionales fue similar a la encontrada en la bibliografía (Xu y Chang, 2007; Vernaza y col., 2012). Los resultados revelan la elevada capacidad antioxidante en mucuna, seguido de caupí. Tras el proceso de germinación se incrementó dicha capacidad, especialmente en caupí (89%) y dolichos (22%). El germinado de mucuna siguió destacando por su alta capacidad antioxidante en ambos métodos. El análisis de correlación estadística revela elevados coeficientes de correlación entre los diferentes métodos utilizados (FRAP r = 0,98 y DPPH r = 0,97, $p < 0,05$).

Por lo tanto, el valor nutricional de estas legumbres tras la germinación mejora debido a la reducción de inhibidores enzimáticos. También cabe destacar un incremento de compuestos fenólicos, así como la capacidad antioxidante. A partir de estos resultados, se planteó en la presente Memoria por una parte, la búsqueda de otras matrices con altas capacidades antioxidantes y por otra parte, la identificación de nuevos compuestos bioactivos que pudieran estar presentes y contribuir sobre esta capacidad.

En este sentido, para conseguir el **segundo objetivo** propuesto, se realizó un "*screening*" de antioxidantes de referencia (patrones), que pueden estar presentes en los germinados, incluyendo la melatonina en dicho estudio, y así avanzar en el conocimiento de sus mecanismos de acción (**Publicación II**). Para ello, se planteó un estudio cinético de los principales antioxidantes presentes en vegetales que aparecen en la bibliografía utilizando el método del DPPH. Se seleccionaron patrones de compuestos fenólicos (ácido gálico, ácido clorogénico, ácido ferúlico, ácido p-coumárico, catequina, kaempferol y quercetina), patrones antioxidantes (ácido ascórbico y trolox) y se incluyó un compuesto indólico (melatonina). En primer lugar, se evaluó el DPPH$^{\bullet}$ remanente hasta el final de la reacción en diferentes concentraciones de trolox y melatonina. El trolox es el principal compuesto antioxidante estudiado para estimar las propiedades antirradicálicas en alimentos debido a su alta solubilidad en agua (Mishra y col., 2012). La melatonina mostró una reacción en estado estacionario hasta los 10 minutos, después reaccionó rápidamente con el DPPH$^{\bullet}$. Ambos compuestos, exhibieron tendencias similares de manera que a mayor concentración mayor fue el porcentaje de DPPH$^{\bullet}$ remanente. Sin embargo, los datos obtenidos revelan que el trolox tiene una mayor actividad antirradical que la melatonina lo que puede ser debido a los distintos grupos funcionales que poseen estos compuestos para atrapar las reacciones (Sharma y Bhat, 2009; Rodríguez-Naranjo y col., 2012). Por lo tanto, estos resultados ponen de manifiesto que las indolaminas no proporcionan adecuados radicales peroxilos para atrapar antioxidantes *in vitro* debido a la ausencia de grupos hidroxilo, sin embargo, pueden ser capaces de atraparlos *in vivo* (Reiter y Tan, 2003). La actividad de la melatonina, a diferencia de otros compuestos antioxidantes, no sólo se basa en su capacidad para eliminar las especies radicales, sino también es capaz de aumentar las actividades de las enzimas antioxidantes y sus productos de degradación son muy eficaces como antioxidantes (AFMK, AMK y 3-hidroximelatonina cíclica (C3-OHM)) (Korkmaz y col., 2009; Reiter y col., 2010).

Para evaluar la capacidad atrapadora del resto de antioxidantes utilizados, también se estudió la cinética a una concentración de 300 µM. Destacó la rápida reacción del ácido ascórbico con DPPH$^{\bullet}$ alcanzando en 5 minutos una respuesta completa con un porcentaje de inhibición similar al Trolox, lo que corrobora los estudios de Sharma y Bhat, (2009). La reacción de DPPH$^{\bullet}$ con melatonina, catequina, kaempferol y trolox

alcanzan el final de la reacción en un tiempo de de 10 a 25 minutos; por último, el resto de antioxidantes, ácido gálico, clorogénico, ácido ferúlico, ácido p-coumárico, y la quercetina alcanzan un estado estacionario dentro de 55-65 min. Por otra parte, el ácido p-coumárico y la melatonina mostraron la actividad antirradical más baja (menor de 10%). Estos resultados sugieren que las diferencias de tiempo en cuanto a la duración de la reacción para alcanzar el estado de equilibrio depende de la naturaleza química del antioxidante lo que corrobora la bibliografía (Rodríguez-Naranjo y col., 2011). Por lo tanto, se desprende que un potente antioxidante no siempre actúa de forma rápida, ya que compuestos menos potentes pueden actuar de forma más eficaz en reacciones biológicas, como sucede con la melatonina.

En otros estudios se ha evaluado la actividad de la melatonina mediante el método DPPH. Se ha observado que la melatonina no es un eficiente donador de H• y además muestra una baja reactividad con este radical (Teixeira y col., 2003; Fagali y Catalá, 2012).

Por tanto, aunque la melatonina no posee una elevada capacidad antirradicálica, su mecanismo de acción muestra un tiempo de reacción corto *in vitro*, por lo que podría estar implicada en cambios metabólicos que se producen durante la germinación, convirtiéndola en un buen antioxidante. En este sentido, se consideró necesaria la determinación de su contenido en otras matrices vegetales para conocer la aportación de este compuesto a la capacidad antioxidante. De esta forma, para cumplir el tercer objetivo propuesto, se determinó el contenido de **melatonina** en otros germinados muy utilizados en la actualidad, siendo la primera vez que se detectaba. El ensayo se realizó con germinados de legumbres y hortalizas comerciales (alfalfa, lenteja, soja verde, cebolla, brócoli, col roja y rabanito), cuyas condiciones de germinación se desconocen por motivos de confidencialidad de la empresa Aunatura Land S.A. La presencia de melatonina se confirmó en todas las semillas de leguminosas y hortalizas analizadas mediante el ensayo de tipo competitivo de ELISA (**Publicación II**). Destaca el contenido de melatonina en brócoli, col roja y rabanito; y similares niveles fueron encontrados en otros alimentos de origen vegetal como tomates (Arnao y Hernández-Ruiz, 2007). Por tanto, se indica la variabilidad de los niveles de melatonina en especies de plantas. Tras el proceso de germinación, se observa que el contenido de melatonina se incrementó en todas las semillas. Los niveles detectados en col roja, rabanito y brócoli fueron muy destacados, por lo que estas semillas germinadas pueden ser consideradas como fuente dietética de melatonina.

Asimismo, se determinó el contenido de **compuestos fenólicos**, y sus familias, cuyos niveles se incrementaron tras la germinación, este hecho corrobora los resultados obtenidos en el primer ensayo así como los encontrados en otras semillas germinadas (Cevallos-Casals y Cisneros-Zevallos, 2010). Como consecuencia del procesado se

pueden producir variaciones en el contenido de las familias de compuestos fenólicos, como son las catequinas y proantocianidinas. De esta forma, las catequinas se incrementaron notablemente tras la germinación, aunque en semillas de soja verde que no fueron detectadas. Estos cambios pueden ser debidos a la presencia de flavonoides, responsables de la activación enzimática (Dueñas y col., 2009).

A continuación, se realizó el estudio cinético en cada uno de los germinados comerciales (**Publicación II**), los resultados indicaron que las semillas que reaccionaron más rápidamente con el DPPH fueron la col roja, alcanzando un tiempo de estabilización en menos de 20 minutos, seguida de lenteja a los 40 minutos, por lo que se considera que éstas tienen un efecto protector rápido. Además, las semillas de col roja, brócoli y rabanito exhibieron una actividad antirradicálica muy importante, alcanzando un porcentaje de inhibición superior al 90%. Tras el proceso de germinación, se observó que sus germinados mostraron una mayor actividad antirradicálica superior al 97%.

Posteriormente, una vez conocida la cinética, se evaluó la **capacidad antioxidante** utilizando tres métodos diferentes: DPPH, FRAP y ORAC para comprender su eficacia como antioxidantes (**Publicación II**). Los resultados obtenidos mediante estas tres metodologías (FRAP, ORAC y DPPH) proporcionan una valiosa información acerca de los diferentes mecanismos implicados. En el caso del FRAP, se basa en una reacción de transferencia de electrones, en el caso del ORAC, se trata de un método de transferencia de protones y el DPPH, puede actuar simultáneamente a través de mecanismos de transferencia de protones y electrones (Huang y col., 2005). Las semillas con mayores contenidos de compuestos fenólicos y melatonina mostraron una alta capacidad antioxidante. Se destaca la semilla de col roja que mostró la mayor capacidad antioxidante en todos los métodos empleados, seguido de brócoli y rabanito. Según la bibliografía, los compuestos fenólicos contribuyen a la actividad antioxidante de las semillas (Xu y Chang, 2007; Gorinstein y col., 2009). Sin embargo, los resultados obtenidos también sugieren que puede ser debido a la presencia de melatonina, que actúa de forma sinérgica con los compuestos fenólicos.

Se pone de manifiesto una alta capacidad antioxidante en todas las semillas germinadas, incrementándose sus niveles hasta 3 veces. Además, las muestras germinadas que presentaron mayor contenido de compuestos fenólicos y melatonina, exhibieron mayor capacidad atrapadora de radicales libre, de manera similar se observaba en las semillas. Por otro lado, los resultados mostraron altas correlaciones ($r = 0{,}90\text{-}0{,}98$, $p < 0{,}01$) entre los compuestos fenólicos y la capacidad antioxidante lo que corrobora la bibliografía existente (Xu y col., 2007; Gorinstein y col., 2009).

En resumen, la germinación provoca modificaciones en los compuestos bioactivos presentes en las semillas, destacando los notables incrementos de la melatonina en los germinados vegetales. Por lo tanto, a pesar de la baja capacidad antirradicálica de este compuesto, su rápido tiempo de reacción *in vitro* podría tener una posible implicación en los cambios metabólicos que tienen lugar en el proceso de germinación, y por lo tanto, contribuir a la capacidad antioxidante de los germinados.

Debido a estos hallazgos, para alcanzar el **cuarto objetivo** propuesto en la presente Memoria se evaluó la influencia de dos condiciones lumínicas (12h luz/12 h oscuridad y 24 h oscuridad) durante un periodo de 8 días de germinación en judía pinta y lenteja con el fin de lograr germinados con altas concentraciones de melatonina (**Publicación III**). Para ello, se empleó otra metodología analítica, concretamente un equipo de HPLC-MS/MS utilizada en la bibliografía para determinar el contenido de **melatonina** en estas legumbres. Los resultados obtenidos indicaron una mayor concentración de melatonina en semillas de judía pinta que en lenteja. El proceso de germinación produce el esperado incremento, siendo éste significativo y dependiente del tiempo. En este ensayo, se detectó el mayor contenido de melatonina en la semilla de lenteja en el sexto día de germinación en ambas condiciones lumínicas; mientras que en judía pinta, se detectó mayor concentración en 24 h en oscuridad. Similares tendencias se obtuvieron en estudios preliminares de Zielinski y col. (2001), observándose que el contenido de melatonina se incrementó en semillas de lenteja, guisante y soja durante 7 días de germinación.

De estos resultados, se concluye que la síntesis de melatonina en matrices vegetales también parece ser dependiente de las condiciones lumínicas a las que se vean sometidas, de igual manera que sucede en humanos, cuyos niveles se encuentran influenciados principalmente por el fotoperiodo como se ha demostrado con numerosos estudios (Reiter, 1993; Brainard y col., 2001; Arendt, 2006; Hardeland y col., 2011). Por otro lado, las similitudes de ambas especies en cuanto al día del valor máximo de melatonina durante la germinación y a la tendencia de incremento de la misma parecen indicar una regulación similar en ambos germinados y que indudablemente, aumenta durante periodos de oscuridad.

En relación a los **compuestos fenólicos**, se llevó a cabo un procedimiento de extracción empleado que proporcionó información acerca del contenido de estos compuestos tanto los que se encuentran libres como unidos a la matriz. Los resultados indicaron que las semillas de lenteja y judía pinta mostraban mayor cantidad de compuestos fenólicos que los germinados, siendo la distribución de un 88% de compuestos fenólicos libres en semillas de lenteja, y de un 92% en judía pinta y como consecuencia, un menor porcentaje de compuestos fenólicos unidos, principalmente ligados a la

pared celular. Estos resultados confirman recientes datos bibliográficos encontrados en otras semillas de leguminosas (Guo y col., 2012).

Al someter dichas semillas al proceso de germinación los niveles de compuestos fenólicos se modifica, en el caso de lenteja se observa un descenso de estos compuestos en ambas condiciones lumínicas (12h luz/12 h oscuridad y 24 h oscuridad), que se inicia tras el tercer día de germinación y continua hasta el sexto día de germinación, alcanzando una reducción global del 72% en condiciones lumínicas de 12 h luz/12h oscuridad; mientras que en condiciones de 24 h oscuridad, dicho descenso sólo llega hasta el 50%. Esto parece ser debido a la presencia de compuestos fenólicos libres en estos germinados y también, al incremento significativo que se produce en compuestos fenólicos unidos (1,6 veces con respecto la semilla de lenteja) bajo 24 h en oscuridad. Con respecto a la judía pinta, similar tendencia se produce, excepto en condiciones de 24 h de oscuridad, la germinación da lugar a un incremento del contenido de compuestos fenólicos totales, tanto libres como unidos, aunque estadísticamente no sea significativo. En consecuencia, estos datos ponen de manifiesto los distintos cambios que se producen bajo las diferentes condiciones lumínicas ensayadas durante el proceso de germinación y corroboran los resultados de otros autores (Khandelwal y col., 2010; Troszyńska y col., 2011). Estas pérdidas observadas pueden ser debidas bien a la unión de los compuestos fenólicos con otros componentes como carbohidratos o proteínas (Khandelwal y col., 2010), o bien a la activación de enzimas endógenas como las hidroxilasas o polifenoloxidasas responsables de la oxidación de compuestos fenólicos endógenos y otros procesos catabólicos en semillas (Świeca y col., 2012).

La capacidad antioxidante (ORAC) de las semillas de este estudio revelaron valores inferiores que los conseguidos por Xu y col. (2007), pero similares a otros estudios (Xu y Chang, 2007; Guajardo-Flores y col., 2013). La capacidad antioxidante en ambas leguminosas se incrementó a lo largo de la germinación (**Publicación III**). Numerosos estudios corroboran que el proceso de germinación provoca un aumento potencial de la capacidad antioxidante (López-Amorós y col., 2006; Dueñas y col., 2009; Guajardo-Flores y col., 2013; Cáceres y col., 2014) y además, las diferentes condiciones de luz durante la germinación influyen significativamente sobre la capacidad antioxidante (Świeca y col., 2012). Los germinados de lenteja presentan mayores incrementos (2,2-2,4 veces) que los germinados de judía con aumentos que oscilan entre 1,6-1,9 veces, siendo mayores en condiciones de oscuridad total.

Es preciso destacar que en el sexto día de germinación la capacidad antioxidante disminuía, correspondiendo con los niveles más bajos de compuestos fenólicos, y con los máximos contenidos de melatonina. Sin embargo, en la etapa final del proceso se produce un aumento significativo de la capacidad antioxidante (octavo día) y una correlación inversa con los obtenidos de melatonina en los germinados ($r = 0,61$-$0,75$, p

<0,01). Este comportamiento puede ser el resultado de las propiedades antioxidantes indirectas de la melatonina, como la activación de cascadas enzimáticas antioxidantes, incluyendo glutatión peroxidasa, superóxido dismutasa y glutatión reductasa y la acumulación de productos de la degradación de gran eficacia (AFMK, AMK y 3-hidroximelatonina cíclica) (Korkmaz y col., 2009; Reiter y col., 2010). Por tanto, los germinados de leguminosas de este estudio ofrecen la posibilidad de ser fuente de antioxidantes naturales que probablemente podrían influir positivamente sobre el estado antioxidante global en sus consumidores.

Debido a la implicación de la melatonina sobre la capacidad antioxidante se realizó el siguiente estudio con la finalidad de conseguir un extracto de germinados de leguminosas rico en melatonina y alto carácter antioxidante, que corresponde con el **quinto objetivo** planteado en la Memoria. Para ello se diseñó un ensayo de germinación, en el que se empleó agua de riego con melatonina (pura) de forma exógena (20 μM), en condiciones de 24 h oscuridad durante un periodo de 10 días (**Publicación IV**). Se seleccionó esta condición lumínica para este ensayo tras la obtención de mejores resultados del estudio anterior.

A pesar de la escasa bibliografía existente sobre melatonina y germinación de alimentos, hay algunos estudios donde la aplicación exógena de melatonina se propone bien como promotora del crecimiento y de la regeneración de raíces o bien, su aplicación en cultivos hidropónicos y su variación al someter a la planta a distintas situaciones de estrés. Los resultados encontrados indican un aumento de los niveles de la neurohormona como resultado del sistema de defensa de la planta frente a situaciones de estrés (Arnao y Hernández-Ruiz, 2007; Zhang y col., 2013).

Por esta razón, se abordó el papel que podía presentar la melatonina presente en los germinados para la promoción del crecimiento de las plantas de forma similar a las auxinas como el ácido indolacético (IAA). En este sentido, se constata que la longitud de la radícula aumenta paulatinamente a medida que la germinación avanza en ambas especies, detectándose mayores longitudes en el caso de judía pinta que en la lenteja (**Publicación III**) en condiciones de 24 h oscuridad. El crecimiento de la radícula mejora significativamente en lenteja y judía pinta con la adición de melatonina de forma exógena, llegándose a incrementar hasta 1,6 veces en el caso de la judía pinta (**Publicación IV**). Por lo tanto, se corrobora que la tasa de germinación y el crecimiento de la radícula puede estimularse o inhibirse (Chen y col., 2009; Park, 2011; Park y Back, 2012; Arnao y Hernández-Ruiz, 2013) y que la adición de 20 μM de melatonina actúa como bioestimulador (Janas y Posmyk, 2013).

Una vez establecido, que la adición de melatonina favorece la germinación, los resultados obtenidos pusieron de manifiesto que la adición de **melatonina** de forma

exógena en el agua de riego incrementa sus niveles en estas leguminosas si se compara con la germinación tradicional. Asimismo, se puede apreciar que el contenido de melatonina en lenteja es mayor que en judía pinta durante el periodo de germinación estudiado. Los niveles de melatonina en lenteja fluctúan durante los primeros días de germinación alcanzando su nivel máximo en el séptimo día, tras el cual comienza a descender. En el caso de judía, se observa un patrón similar al mostrado en la lenteja, detectándose el nivel más elevado en el sexto día de germinación.

A pesar de que la semilla de lenteja tiene una concentración inicial menor, ésta fue capaz de absorber hasta un 24% de la melatonina añadida, lo que dió lugar a mayores niveles de melatonina tras la germinación; mientras que en la semilla de judía pinta el contenido inicial de melatonina es mayor, pero sólo absorbió un 11% por consiguiente, los niveles de melatonina alcanzados fueron menores. Es preciso señalar, en el caso de la lenteja, ésta presenta una cubierta más delgada entre 25-35 μm (8% del total de la semilla) dependiendo del cultivo (Tiwari y Singh, 2012), lo que facilitaría la absorción de melatonina mientras, la judía exhibe una cubierta más gruesa entre 40-60 μm (12% del total de la semilla) (Tiwari y Singh, 2012). En consecuencia, la cubierta de la semilla de judía pinta no permite la difusión de melatonina tan fácilmente como la lenteja. A pesar de que la lenteja presenta mayores niveles de melatonina, la judía pinta tiene un mayor rendimiento de germinación (89%), lo que implica menores pérdidas durante el proceso y por lo tanto, resulta más eficaz.

A la vista de los resultados obtenidos en estos ensayos, se pone de manifiesto que en la oscuridad se facilita la síntesis de la melatonina y que esta condición, junto a la aplicación de melatonina exógena produce un incremento drástico de los contenidos de este componente en las muestras vegetales estudiadas. Este hecho tiene consecuencias muy positivas tal y como indica la bibliografía, puesto que la metabolización de la melatonina conduce a relevantes incrementos de la actividad antioxidante de algunas enzimas (Tan y col., 2000; Reiter y col., 2005). Las semillas tienen mayor contenido de lípidos que otras partes de la planta, ya que poseen altos niveles de ácidos grasos poliinsaturados. Durante la germinación, los lípidos pueden ser metabolizados por β-oxidación y gluconeogénesis para la obtención de energía, provocando un aumento de ROS (Schopfer y col., 2001; Perl-Treves y Perl, 2002). El posible papel de la melatonina como antioxidante, puede reducir los niveles de ROS y favorecer la regulación del crecimiento y desarrollo de la planta durante la germinación (Kim y Cho, 2011).

Los mayores contenidos de melatonina encontrados en lenteja condujeron a un estudio más detallado de los niveles de esta molécula en los diferentes tejidos de la semilla germinada (cutícula, endospermo y radícula) bajo las mismas condiciones de germinación anteriores (10 días y 24 h oscuridad) (**Publicación IV**). Tras el tercer día

de germinación se detectaron mayores niveles de melatonina en la cubierta de la semilla que en el resto de tejidos (endospermo y radícula), aumentando su concentración en todos los tejidos hasta el octavo día de germinación. Hasta la fecha, no existen estudios relativos a la concentración de melatonina en los diferentes tejidos de semillas germinadas, por lo que estos resultados son de interés ya que ofrecen la posibilidad de conocer el posible papel protector de la melatonina sobre el estrés oxidativo en dichos tejidos.

Por otro lado, las modificaciones producidas en los niveles de **compuestos fenólicos** en lenteja y judía pinta son dependientes del tiempo de germinación. Así, la lenteja muestra descensos en los compuestos fenólicos libres y aumento de los compuestos fenólicos unidos (17%), posiblemente debido a la glicosilación de compuestos fenólicos libres producida por la activación de enzimas (Modolo y col., 2007). En el caso de la judía pinta, no se observan descensos significativos tanto en compuestos fenólicos totales como en compuestos fenólicos libres. Diferentes investigaciones revelan que las condiciones de iluminación pueden inducir la vía pentosa fosfato para la síntesis de compuestos fenólicos (Shetty, 2004; Khattak y col., 2007). Aunque se han llevado a cabo procesos de remojo previos a la germinación con concentración de melatonina de 20μM (Posmyk y col., 2008; Szafrańska y col., 2012), no existen estudios en la actualidad sobre los posibles cambios que sufren los compuestos fenólicos en estas condiciones. Sin embargo, otros antioxidantes han sido utilizados como agua de riego en semillas, por ejemplo, el ácido ascórbico en semillas de haba, observándose un aumento de la actividad enzimática de la peroxidasa, lo que implicaba cambios en la pared celular (Randhir y col., 2004). Asimismo, la presencia de elicitores pueden estimular diferentes clases de compuestos fenólicos y afectar a su concentración siendo éstos más dependientes de la genética de la planta que de la naturaleza química del elicitor (Dueñas y col., 2015). Por lo tanto, los resultados obtenidos sugieren que existe un catabolismo de compuestos fenólicos para la biosíntesis y reorganización de la pared celular durante la germinación en respuesta a la adición de melatonina.

Como se ha comentado anteriormente, la germinación produce un aumento de la **capacidad antioxidante** en ambas especies de leguminosas, lenteja y judía pinta. Sin embargo, mayores incrementos fueron detectados con la adición de melatonina exógena (**Publicación IV**). Se corrobora que la lenteja exhibe mayor capacidad antioxidante que la judía pinta, incrementándose hasta 1,7 veces tras la germinación. La adición de melatonina podría ser la causa de este aumento en la capacidad antioxidante como consecuencia de las reacciones indirectas que desencadena la presencia de melatonina sobre el estrés oxidativo, como se ha citado anteriormente (Tan y col., 2000; Reiter y col., 2005). Además, la melatonina presenta una correlación positiva con la capacidad antioxidante en ambos germinados, especialmente en el caso de lenteja ($r = 0,855$, $p < 0,01$).

Debido a que la melatonina y los compuestos fenólicos poseen relevante capacidad antioxidante, se realizó la determinación de la capacidad antioxidante en los diferentes tejidos estudiados de lenteja (cutícula, endospermo y radícula). Los resultados indicaron que la cubierta de la semilla posee la mayor capacidad antioxidante y como se esperaba, el endospermo mostró una capacidad antioxidante baja a lo largo de la germinación. La radícula exhibe 3 veces más capacidad antioxidante que el endospermo, pero no se aprecian incrementos significativos durante el proceso. Asimismo, los contenidos de melatonina revelan una correlación muy alta con la capacidad antioxidante en el endospermo de la lenteja y sus germinados ($r = 0,902$ y $r=0,855$, respectivamente, $p < 0,01$).

A partir de los resultados y discusión de los ensayos realizados en relación al efecto de la germinación sobre matrices vegetales fuente de compuestos bioactivos, se puede concluir que este proceso se contempla como una técnica sencilla, fácil de aplicar y con un mínimo coste. En cuanto a los compuestos bioactivos que se han evaluado, este proceso produce un incremento de melatonina en todos los germinados de leguminosas y hortalizas estudiadas. Del mismo modo, puede incrementar o reducir el contenido de compuestos fenólicos, dependiendo de la especie, variedad y condiciones de germinación. Asimismo, en todos los germinados estudiados, la capacidad antioxidante se ve incrementada y la melatonina contribuye a ello, debido a su potente acción antioxidante por su alta eficacia y papel protector frente a los radicales libres (ROS) y las especies reactivas de nitrógeno (RNS). También es capaz de estimular la actividad y expresión de otros sistemas antioxidantes, protegiendo del daño oxidativo por vía indirecta, a través de la activación de enzimas antioxidantes. No sólo la melatonina sino sus metabolitos son eficaces en reducir el daño producido por radicales libre, dando lugar a la cascada antioxidante. Por todo ello, la germinación puede ser considerada como un procedimiento efectivo para incrementar la bioactividad de leguminosas y hortalizas, aunque es necesario realizar más estudios *in vitro* e *in vivo* para conocer el potencial real de estos germinados como ingredientes en la dieta.

Por esta razón, el **sexto objetivo** de esta Memoria, fue conocer la biodisponibilidad de melatonina presente en un extracto de judía germinada mediante un ensayo *in vivo* en animales de laboratorio. Se investigó la influencia de la ingesta de un extracto rico en melatonina obtenido a partir de judías germinadas (sexto día de germinación), obtenidos en la Publicación IV, sobre la concentración de la misma en sangre y otros componentes de las rutas de síntesis y degradación de melatonina en orina (*Rattus norvegicus*), así como su posible efecto sobre la capacidad antioxidante en plasma (**Publicación V**). Se llevaron a cabo dos experimentos, en el ***experimento 1***, a la edad de 45 días, las ratas se sometieron a diferentes condiciones de alimentación realizándose diferentes grupos: E1.i) pienso estándar para ratas sin extracto (control); E1.ii) 24 h

período de ayuno (ayuno); E1.iii) administración de extracto de judía después de 24 h de ayuno, seguido en todos los casos por el muestreo de sangre y orina. En el ***experimento 2***, las muestras se obtuvieron del mismo grupo de ratas los grupos fueron: E2.i) después de una alimentación común (Control); E2.ii) tras 12 h en ayunas (en ayunas); E2.iii) después de 12 h (en ayunas)+ la ingesta del extracto de judía (JP Extracto), y finalmente el experimento E2.iv) tras la ingesta de una solución de melatonina pura (MEL).

En el ***experimento 1***, se constató que la **melatonina** presente en los germinados de judía fue absorbida y se incrementó el nivel plasmático de este componente; sin embargo, los resultados no llegaron a ser tan elevados como los niveles de melatonina observados después del consumo de nueces (Reiter y col., 2005). La oscilación de los niveles plasmáticos de melatonina podría ser debida a diferentes factores entre ellos, edad de los animales, cantidad de melatonina ingerida y el período de administración (de horas a semanas), etc. (Reiter y col., 2005; Delgado y col., 2012; Sae-Teaw y col., 2013). Además, el tiempo de extracción de la sangre puede influir si está cerca de la acrofase (Delgado y col., 2012). Recientes estudios han demostrado que el consumo de diferentes frutas conducen a variaciones en los niveles plasmáticos de melatonina debido a su diferente biodisponibilidad (González-Flores y col., 2012; Sae-Teaw y col., 2013; Johns y col., 2013).

Asimismo, se analizó el contenido de **serotonina**, precursor de la melatonina, tras la ingesta del extracto después de 90 minutos se produce un incremento importante de los niveles de serotonina. Por lo tanto, la influencia de la dieta parece relevante, después de 90 min de su administración en los niveles de serotonina, que alcanzaron el 65% más del grupo de control.

Otro compuesto, el 6-sulfatoximelatonina urinario (**aMT6s**), fue determinado para conocer la biodisponibilidad de la melatonina. Este compuesto sulfatado es uno de los principales metabolitos de melatonina en la orina y es considerado como un buen indicador de la concentración de melatonina en plasma, ya que muestra una correlación plasmática (Johns y col., 2013). Se detectó un incremento drástico de los niveles de aMT6s (4 veces superior) después de la ingesta del extracto de judía, en comparación con el contenido basal y los niveles en ayunas. Los resultados corroboraron que la ingesta del extracto anterior produce aumentos significativos similares a los obtenidos tras la ingesta de verduras y frutas (González-Flores y col., 2012; Johns y col., 2013). Los niveles de aMT6s encontrados después de la ingesta de extracto de judía ponen de manifesto la alta eficiencia catalítica de sulfatación melatonina en ratas, puesto que la melatonina se metaboliza rápidamente a 6-hidroximelatonina la cual es rápidamente conjugada a aMT6s (Zhao y col., 2016). Del mismo modo, la tasa de sulfatación intrínseca total de melatonina presenta diferencias

considerables entre especies, siendo mayor en ratas que en los seres humanos o ratones (Tian y col., 2015). En consecuencia, el nivel de aMT6s aumenta rápidamente después de la ingesta del extracto de judía rico en melatonina.

Con respecto a los **compuestos fenólicos** plasmáticos, los tres grupos estudiados mostraron niveles estadísticamente similares (1 mg GAE/ mL). El tiempo de vida media plasmática de estos compuestos es de 2 a 8 h, pero en ocasiones puede alcanzar hasta 12-24 h (Manach y col., 2004). Se ha demostrado que los niveles plasmáticos de compuestos fenólicos generalmente, exhiben una aguda disminución una hora después de la ingestión (Henning y col., 2004; Velderrain-Rodríguez y col., 2014). Sin embargo, en este estudio los niveles de compuestos fenólicos en plasma de ratas tras 24h de ayuno no indicaron cambios, probablemente debido a su acumulación en el plasma, o en otros tejidos, como han revelado previamente algunos estudios (Moon y col., 2000). Su presencia en el intestino, principalmente en el intestino grueso, en mayor cantidad que en plasma, parece ser la causa principal del mantenimiento constante de los compuestos fenólicos totales (Velderrain-Rodríguez y col., 2014). Debido a que la microbiota del colón está implicada en la formación de ácidos fenólicos procedentes de complejos poliméricos de naturaleza fenólica mediante procesos de hidrólisis de glucósidos, fisión y oxidación de anillos fenólicos, los metabolitos resultantes pueden ser absorbidos e introducirse en la circulación sistémica. En consecuencia, la medida de compuestos fenólicos después de 24 horas de ayuno podría indicar, en cierta medida, el contenido de todos estos compuestos, procedentes de la dieta de las ratas, principalmente de cereales (trigo, maíz, salvado de trigo, cebada, soja, etc.). Por otra parte, como la ingesta de extracto de judía pinta se realizó en un periodo de tiempo corto, los compuestos fenólicos podrían no haber alcanzado la etapa de digestión, y por tanto, no estar biodisponibles.

Para evaluar la **capacidad antioxidante**, se seleccionaron dos ensayos (ORAC y FRAP) con diferentes mecanismos antioxidantes. Los resultados obtenidos mediante ORAC resultaron ser similares entre sí, sin diferencias significativas. De la misma manera sucede con los niveles de antioxidantes FRAP entre los grupos estudiados. Por tanto, estos resultados parecen indicar que la presencia de melatonina en los extractos de judía pinta no influyen de manera directa en el estatus antioxidante del plasma de las ratas. Los ensayos FRAP y ORAC sólo miden la capacidad de captación de radicales libres y la melatonina expone acciones antioxidantes directos e indirectos (Korkmaz y col., 2009; Bonnefont-Rousselot y Collin, 2010) incluyendo la estimulación de expresión de enzimas antioxidantes endógenos (Rodríguez y col., 2004). La capacidad antioxidante tras una sola administración de extracto no puede detectar los efectos antioxidantes indirectos de la melatonina. Para ello, hubiera sido necesario realizar la administración del extracto de judía durante un periodo de tiempo más largo para conocer dichos efectos. Además, es posible que los resultados obtenidos revelen la

estimación de niveles de otros antioxidantes como compuestos fenólicos y por tanto, no se pueden atribuirse exclusivamente a los contenidos de melatonina. Como se ha demostrado en otros estudios, la ingesta de alimentos ricos en melatonina tales como cerezas, zumos de uva o cerveza produjeron un aumento de la capacidad antioxidante plasmática (Maldonado y col., 2009; Garrido y col., 2010; González-Flores y col., 2012). Sin embargo, estos resultados podrían no estar relacionados directamente con los niveles de melatonina tal y como sucede en nuestro estudio. Del estudio de correlación lineal de Pearson sólo el nivel de aMT6s en la orina se correlacionó con el nivel plasmático de melatonina ($r = 0{,}713$, $p < 0{,}01$).

En cuanto al ***experimento 2***, cabe indicar que es la primera vez que se realiza un estudio sobre la biodisponibilidad de la melatonina que se encuentra en una matriz alimentaria comparándola con una solución de melatonina pura. Para conseguir este propósito, un cuarto grupo de ratas se incluyó en este experimento, administrando el mismo contenido de melatonina presente en el extracto de judía pinta como de melatonina sintética (MEL) (10,6 μg). Los niveles de **melatonina** en el cuarto grupo (MEL) fueron significativamente más elevados, en comparación con el resto de los grupos. Por lo tanto, estos resultados ponen de manifiesto que la mayor biodisponibilidad de melatonina se produce en la solución acuosa (17% de aumento) frente a la matriz del extracto de judía pinta. A partir de estos resultados, se puede deducir que la influencia de la matriz afecta directamente a los niveles de melatonina en plasma sanguíneo. La melatonina en el extracto de judía pinta está presente junto a otros compuestos solubles en metanol, como los compuestos fenólicos, que provocan cambios en su biodisponibilidad, como se ha descrito en otros estudios relacionados con la evaluación de la ingesta dietética de melatonina a partir de frutas (Johns y col., 2013).

En cuanto a la **capacidad antioxidante**, se confirma el comportamiento observado en el experimento 1, ya que los resultados obtenidos fueron similares en todos los grupos, incluido el grupo MEL. Por lo tanto, las variaciones en los niveles plasmáticos de melatonina no provocaron diferencias en la capacidad antioxidante.

En conclusión, en este estudio se ha demostrado que los germinados de judía pinta que contienen un importante contenido de compuestos antioxidantes bioactivos, especialmente compuestos fenólicos, constituyen el principal fitoquímico antioxidante que se encuentra en el plasma. Asimismo, la melatonina está disponible en plasma tras la ingestión de germinados de judía pinta en ratas sometidas 24 horas de ayuno. La capacidad de captación de radicales libre en plasma tanto en ratas a las que se les ha suministrado extracto de judía pinta o bien melatonina pura es similar, probablemente debido a los efectos de enmascaramiento de los compuestos fenólicos. Sin embargo, es posible que la administración de alimentos ricos en melatonina durante un periodo de

tiempo más prolongado elevará sus contenidos en plasma, pudiendo ejercer acciones antioxidantes indirectas, como se ha descrito ampliamente con melatonina sintética en ensayos clínicos. Hasta la fecha, los beneficios para la salud derivados de la ingesta dietética de la melatonina son, hasta ahora, controvertidos, ya que no se reconoce si el consumo crónico de la melatonina a través de la dieta posee efectos fisiológicos.

Por lo tanto, las legumbres germinadas pueden ser una fuente natural adecuada de melatonina exógena si se consumen de manera habitual. Sin embargo, más investigaciones clínicas al respecto son necesarias realizar para determinar si el consumo de melatonina a largo plazo en la dieta produce efectos positivos en los sistemas de defensa antioxidante y por tanto, puede intervenir en la prevención de enfermedades.

Sugerencias de futuras investigaciones

Como futuros estudios dentro de esta temática y en la búsqueda de nuevas fuentes de melatonina, se ha comenzado a trabajar en una nueva línea de investigación en la que destacamos las infusiones de hierbas medicinales comúnmente consumidas en Europa (Anexos). Los niveles de melatonina, compuestos fenólicos así como la capacidad antioxidante han sido evaluados, además del efecto inhibidor *in vitro* frente a las enzimas relacionadas con el metabolismo energético, como son la lipasa, α-glucosidasa y α-amilasa. Por otro lado, también se ha estudiado la presencia de melatonina en café y subproductos, como es la cascarilla de café como aprovechamiento de residuos procedentes de la industria alimentaria, y posible introducción en el consumo como infusión con alto contenido en melatonina (presentación a congreso 3 y 4).

Conclusiones

V. CONCLUSIONES

De la presente Memoria se deducen las siguientes conclusiones:

1. La germinación modifica el perfil de los componentes bioactivos, siendo dependiente del tipo de legumbre y de las condiciones de germinación. En consecuencia, los germinados se pueden considerar como fuente dietética de antioxidantes, tales como la melatonina.

2. Todas las semillas de legumbres y hortalizas comerciales estudiadas contienen melatonina, destacando los niveles en brócoli. No existen datos previos a los descritos en la presente memoria.

3. Los contenidos de melatonina y compuestos fenólicos (totales, catequinas y proantocianidinas) en germinados comerciales fueron significativamente (p<0,05) más elevados que los encontrados en sus respectivas semillas, con la excepción de los germinados de brócoli, lenteja y alfalfa. Los germinados comerciales de col roja resultan ser la mejor fuente dietética de antioxidantes de todas las muestras analizadas.

4. Las condiciones lumínicas durante el proceso de germinación (12h luz/12h oscuridad y 24 h oscuridad) afectan a la síntesis de melatonina en plantas. Los niveles máximos de melatonina en lenteja y judía pinta se alcanzan bajo condiciones de 24 horas en oscuridad en el sexto día de germinación. Los contenidos más elevados se alcanzan en germinados de judía pinta.

5. El tratamiento combinado de riego suplementado con melatonina pura y germinación en oscuridad incrementa su valor absoluto en ambas legumbres, lenteja y judía pinta. El aumento del contenido de melatonina en los germinados puede deberse a fenómenos de adsorción y/o absorción. Se observa crecimiento de la radícula, lo que sugiere un efecto bioestimulador por absorción de melatonina.

6. En la cubierta de la semilla de lenteja germinada se concentra el mayor contenido de melatonina y capacidad antioxidante en comparación con el resto de los tejidos vegetales analizados (radícula y endospermo).

7. La melatonina presente en un extracto de judía pinta administrada en una dosis única (10,6 μg) en ratas Sprague Dawley mediante sonda nasogástrica se encuentra biodisponible. Los niveles de melatonina plasmáticos se incrementan de 25 a 30 pg/mL, indicando una contribución dietética de este

compenente. Los valores de excreción de 6-sulfatoximelatonina (aMT6s), metabolito de degradación de melatonina, incrementan en un orden de cuatro veces tras la ingesta del extracto de judía pinta, indicando su absorción y metabolismo.

Como conclusión general, los resultados de esta Memoria indican que los germinados de legumbres contienen cantidades de melatonina superiores a las descritas en otras matrices vegetales. Sin embargo, son necesarios más estudios clínicos para determinar la biodisponibilidad de la melatonina dietética en germinados de legumbres en humanos y verificar su potencial como fuente dietética de este compuesto; así como, su contribución a la defensa antioxidante *in vivo*.

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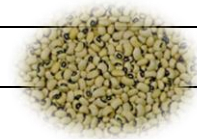
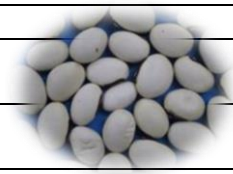
Anexos

VII. ANEXOS

A continuación, se realiza una breve descripción de las muestras utilizadas:

- **Leguminosas no convencionales *(*Publicación I*)***

Vigna unguiculata	
Nombre común	fríjol caupí, cowpea, cabecita negra, etc 
Origen	África tropical
Descripción planta	Es una planta herbácea anual, erecta o trepadora, de hojas verdes trifoliadas, grandes y vigorosas. Posee flores racimosas, vainas largas curvadas de 10 a 23 cm y de 10 a 15 semillas por vaina.
Semillas	Existen semillas de diferentes colores: blanco, rojo, marrón y crema
Características de cultivo	Toleran suelos pobres en nutrientes y elevadas condiciones de acidez
Usos	La semilla es muy utilizada para la alimentación humana y se considera una alternativa de alimentación para el ganado. Se suele utilizar como un ingrediente en la dieta de las aves de corral, cerdos, etc… y además se han desarrollado recetas con harinas utilizadas en tartas, galletas, etc…

Canavalia ensiformis	
Nombre común	Jack bean 
Origen	India y Centroamérica
Descripción planta	Es una planta anual, enredadera de hasta 1 metro de altura. Las flores son de color rosa-púrpura y las vainas son planas de hasta 36 centímetros (consideradas entre las más grandes de todas las leguminosas) con grandes semillas blancas. Las vainas verdes inmaduras son normalmente consumidas como vegetales
Semillas	Grandes semillas blancas
Características de cultivo	Es una especie de clima tropical seco
Usos	

Mucuna pruriens	
Nombre común	Stizolobium niveum, frijol de terciopelo, pica dulce, etc…
Origen	Tropical, distribuidos en África tropical, India y Caribe
Descripción planta	Es una planta anual, rastrera y trepadora. Las flores y vainas están cubiertas de pelos anaranjados, causantes de severa hinchazón y alergia si se ponen en contacto con la piel
Semillas	Blancas, o púrpuras
Características de cultivo	Tiene un desarrollo inicial lento, moderada resistencia a encharcamientos y a largos períodos de sombra, pero es susceptible a heladas
Usos	Para el tratamiento de varias enfermedades provocadas por radicales libres, tales como artritis reumatoide, diabetes, aterosclerosis, infertilidad masculina y trastornos nerviosos. Estas semillas son muy utilizadas en el tratamiento de Parkinson, ya que son una buena fuente de L-Dopa, que es el principal componente fenólico encontrado en estas semillas (5%), aunque también es considerado como un potencial compuesto no deseado

Lablab purpureus	
Nombre común	Lablab purpureus
Origen	Sudeste asiático
Descripción planta	Planta herbácea, anual o bianual, en casos excepcionales perenne; tipos rastreros o semi-erectos: hojas grandes trifoliadas y vigorosas, las flores son en racimo de color blanca o violáceo, vainas cortas de 3 a 4 cm.
Semillas	Ovales de varios colores (de 2 a 6 semillas por vaina y son de color negruzco o pardo
Características de cultivo	No tolera la inundación ni el fuego, pero soporta temperaturas bajas durante un periodo corto. Se cultiva como planta ornamental, sobre todo en EE.UU
Usos	

- **Germinados comerciales (Publicación II)**

Se utilizaron tres variedades de leguminosas: alfalfa (*Medicago sativa* L.), lenteja (*Lens sculenta* L.), y soja verde (*Vigna radiata* L.), y cuatro variedades de hortalizas: cebolla (*Allium cepa L.*), brócoli (*Brassica oleraceae* L.), col roja (*Brassica oleraceae capitata rubra* L.) y rabanito (mezcla de las siguientes variedades: *Raphanus sativus japonicum L., Raphanus sativus rambo* L., *Rhapanus sativus sinicum rosae* L.). Las muestras de legumbres y hortalizas, tanto crudas como procesadas mediante el tratamiento de germinación, fueron suministradas por gentileza de la empresa Aunatura Land S.A (Torres de la Alameda, Madrid). Todas las muestras estudiadas son productos comercializados actualmente por dicha empresa.

Semilla y germinado de Alfalfa
***Medicago sativa* L.**
ORIGEN: Toscana, Italia

Semilla y germinado de Lenteja
***Lens sculenta* L.**
ORIGEN: Canadá

Semilla y germinado de Soja Verde
***Vigna radiata* L.**
ORIGEN: China

Semilla y germinado de Brócoli
***Brassica oleracea* L.**
ORIGEN: Toscana, Italia

Semilla y germinado de Col Roja
***Brassica oleraceae capitata rubra* L.**
ORIGEN: Toscana, Italia

Semilla y germinado de Rabanito
Mezcla de variedades:
***R. sativus japonicum* L.,**
***R. sativus rambo* L.,**
***R. sativus sinicum rosae* L.**
ORIGEN: Toscana, Italia

Semilla y germinado de Cebolla
***Allium cepa* L.**
ORIGEN: Toscana, Italia

- **Germinados bajo condiciones lumínicas y riego con melatonina (Publicaciones III y IV)**

Para la realización del estudio se utilizaron dos variedades de leguminosas:

- Lenteja (*Lens sculenta* L., familia Fabaceae, Variedad Salmantina)
- Judía (*Phaseolus vulgaris* L., familia Fabaceae, Variedad Pinta)

Las muestras de legumbre cruda fueron suministradas por gentileza de la Dra. Frías, del Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN, CSIC).

A) Lenteja (*Lens sculenta* L.);

B) Judía (*Phaseolus vulgaris* L.)

- **Animales de experimentación** (**Publicación V**)

Ratas macho (*Rattus norvegicus*) de la cepa Sprague Dawley, criadas en el animalario de la Facultad de Medicina de la Universidad Autónoma de Madrid y facilitadas por gentileza del grupo de investigación dirigido por la Dra. González del Departamento de Fisiología. Todas ellas se caracterizan por poseer el ciclo cronobiológico cambiado, es decir, desde las 4:00 hasta las 3:59 h se exponen a luz artificial mientras que desde las 4:00 hasta las 15:59 h se mantienen en oscuridad. En definitiva, las ratas se expusieron a 12 h de luz y 12 h de oscuridad. La entrada de las ratas a la cámara de fotoperiodo cambiado se realiza a los 21 días de edad, momento en que se procede al destete de las crías, manteniéndose en ella 15-20 días, tras los cuales se realiza el experimento.

La alimentación habitual es pienso completo para ratas/ratones de laboratorio en fase de crecimiento, en forma de pellets y agua ad libitum a excepción de los experimentos explicados en la Memoria. El pienso básico con el que se alimentan las ratas de forma habitual se compone de trigo, maíz, salvado de trigo, cebada, granos de soja extruídos, extracción de terrones de soja, proteínas de pescado hidrolizado, levadura de cerveza, premezcla de vitaminas y minerales, carbonato de calcio, fosfato dicálcico.

Macronutrientes	**%**	**Aditivos**	
		Vitaminas	
Proteínas	21,4	Vit. A (E672) Vit. D3 (E671) Vit E (E307)	15260 IU/Kg 2380 IU/Kg 31 mg/Kg
Lípidos	5,1	Oligoelementos	
Cenizas	5,7	E4 ($CuSO_4.5H_2O$)	15 mg/kg
Fibra	4,0	E1 ($FeSO_4.7H_2O$)	188 mg/Kg
Ca	0,90	E5(MnO_2)	38 mg/Kg
P	0,59	E6 (ZnO)	31 mg/Kg
Na	0,28	E3 ($CoCO_3$)	1,3 mg/

El sondaje para alimentar a los animales con los extractos lo lleva a cabo el personal autorizado del Animalario de la Facultad de Medicina, y para ello, se utiliza una sonda de 8 cm de longitud aproximadamente, de metal, rígida que presenta uno de los extremos redondeado con una luz de 1 mm, y que es acoplado a una jeringuilla. Se introduce dicha sonda por la boca hasta el estómago teniendo el animal completamente inmovilizado de forma que la cabeza, el cuello y el tórax formen una línea recta. Se avanza suavemente hasta alcanzar el estómago y se introduce el contenido total del extracto (2,5 mL). Tras el sondaje se esperaron 90 minutos para que

se produjera la digestión y, a las 10:30 h se realizó la extracción de sangre de todos los individuos.

Para la obtención de muestras biológicas se procedió según la Directiva Europea 86/609/CE de la Unión Europea de 1986, el Convenio ETS 123 del Consejo de Europa de 1986 sobre los animales vertebrados utilizados para experimentación y otros fines científicos, ratificada por España en 1990 y cuya revisión se aprobó en 2006, así como la Ley del Estado Español 32/2007 de Cuidado de los Animales y el Real Decreto 1201/2005 español sobre protección de los animales utilizados en experimentación y otros fines científicos (BOE 21 de Octubre de 2005). El protocolo seguido en todos los ensayos se describe a continuación.

Plasma: En primer lugar, se introduce al animal en la cámara de CO_2 durante unos minutos para su aturdimiento. Las cantidades administradas de este gas son superiores al 60% lo que produce inconsciencia por causa del efecto anestésico de dichas concentraciones sobre el cerebro. A continuación, se procede a la punción cardiaca con una jeringuilla de 5 mL para la obtención del máximo volumen sanguíneo posible directamente desde el corazón. La sangre se traspasa a un eppendorf que contienen 20 µL de heparina al 5% hasta un volumen de 1 mL. Se agita por inversión y se deposita en hielo hasta su posterior centrifugación. Las condiciones de ésta son de 4500 rpm, 10 minutos, 4 °C. Tras la extracción de las muestras de sangre, el animal vuelve a ser introducido en la cámara de CO_2 para su eutanasia.

Orina: La obtención de las muestras de orina se lleva a cabo depositando el animal encima de una tira de parafilm durante el aturdimiento en la cámara de CO_2 y a continuación se recogen las gotitas de orina con una jeringuilla. Las muestras se guardan en eppendorf rodeados de papel aluminio para evitar la luz directa y se almacenan en nieve carbónica para su congelación.

A continuación, se presenta la publicación (enviada) que se hace referencia en el apartado de Discusión general, como propuestas futuras.

Cover Letter

María Ángeles Martín-Cabrejas
Instituto de Ciencias de la Alimentación
Facultad de Ciencias
Universidad Autónoma de Madrid
28049 Madrid (Spain)
Tel 34-914978678
email: maria.martin@uam.es

30th March, 2016

Dear Editors-in-Chief: N. Belgacem, M.T. Berti, E. Frollini, M.J. Pascual-Villalobos,

Enclosed the entitled article *Antioxidants of widespread herbal infusions in Europe and their potential effects on the inhibition of lipase, α-amylase and α-glucosidase* prepared by Teresa Herrera, Yolanda Aguilera, Miguel Rebollo-Hernanz, Elena Bravo, Vanesa Benítez, Nuria Martínez-Sáez, Silvia M. Arribas, María Dolores del Castillo, María A. Martín-Cabrejas.

The relevance of the subject matter to research in *Industrial Crops and Products* has been to provide new knowledge on the profile of the bioactive compounds and health promoting properties of nineteen highly consumed herbal infusions. To achieve the goal the content of melatonin and phenolic compounds, antioxidant capacity, and *in vitro* inhibitory effect against energetic metabolic enzymes named lipase, α-glucosidase, and α-amylase were determined. These infusions are rich in melatonin, phenolic compounds, isoflavones, and inhibitors of lipase and α-glucosidase. Correlation studies seem to indicate phytochemicals contribute to the antioxidant, antidiabetic, and antiobesity potential effects of these infusions. The health benefits derived from the intake of these infusions are, until now, uncertain as it is not recognized if their chronic consumption through the diet have physiological effects, thus further studies are required.

Looking forward to receiving news from you.

Sincerely,

María A. Martín-Cabrejas

Graphical Abstract

Graphical abstract

***Highlights (for review)**

Highlights

- Melatonin and phenolics higher levels were in chamomile and lemon balm, respectively
- Lemon balm, green tea, and boldo infusions showed high antioxidant capacity
- Boldo showed a pronounced inhibition of pancreatic lipase
- Multiple herbal infusions exhibited antidiabetic potential inhibiting α-glucosidase
- Few infusions presented inhibition against α-amylase

Antioxidants of widespread herbal infusions in Europe and their potential effects on the inhibition of lipase, α-amylase and α-glucosidase

Teresa Herrera,[a] Yolanda Aguilera,[a] Miguel Rebollo-Hernanz,[a] Elena Bravo,[a] Vanesa Benítez,[a] Nuria Martínez-Sáez,[a] Silvia M. Arribas,[b] María Dolores del Castillo,[a] María A. Martín-Cabrejas[a]*

[a] Dpto. Química Agrícola y Bromatología, Facultad de Ciencias, Universidad Autónoma de Madrid, Instituto de Investigación de Ciencias de la Alimentación (CIAL). Spain.

[b] Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Spain.

* E-mail: maria.martin@uam.es Tel.: 0034 91 001 7913 Fax: 0034 91 497 3826

Abstract

Levels of melatonin, phenolic compounds, as well as, the antioxidant (DPPH, ABTS, FRAP, ORAC, and deoxyribose assays), antiobesity, and antidiabetic effects of nineteen infusions commonly consumed in Europe were analyzed. Chamomile and lemon balm showed the highest values of melatonin (298 ng /g DW) and phenolic compounds (133 mg/ g DW), respectively. Lemon balm also exhibited the highest flavonoid content (127 mg/ g DW). In agreement, the above infusion showed the highest antioxidant capacity measured by four of the fifth antioxidant methods employed in the present study. Boldo and green tea contain effective enzymatic inhibitors pinpointing their potential for reducing the risk of chronic diseases related to metabolic disorders. The statistical analysis of the data corroborates lemon balm, green tea and boldo are richest sources of bioactive compounds such as flavonoids and phenolic compounds which have a significant contribution ($p < 0.01$) to the health promoting effect hereby ascribed to these infusions.

Keywords

herbal infusion; melatonin; antioxidant capacity; lipase; glucosidase; amylase

17 **1. Introduction**

18 Consumption of herbal infusions in European countries has increased in the last
19 decades (Hoffman and Gerber, 2013). Medicinal plants are widely used in
20 alternative medicine for the prevention of some human diseases since long time
21 ago, and nowadays, consumers are becoming increasingly interested because of
22 their health benefits and minimum side effects. Studies indicated that medicinal
23 plants possess more potent antioxidant activity than common fruits and
24 vegetables (Cai et al., 2004). Therefore, antioxidant compounds present in
25 medicinal plants can be used for inhibiting or preventing diseases consequence of
26 oxidative stress (Li et al., 2013). This is one of the main factors in the
27 development of metabolic syndrome, characterized by glycaemic index
28 imbalance, glucose intolerance, hypertension, dyslipidaemia, and/or obesity (Sen
29 et al., 2013).

30 Polyphenolic-rich extracts obtained from herbs have been reported to modulate
31 the activity of selected digestive enzymes, such as α-amylase, α-glucosidase, and
32 lipase, involved in energetic metabolism (Grove et al., 2012; Matsui et al., 2001;
33 McDougall et al., 2009). α-Glucosidase and α-amylase inhibitors were found
34 naturally in medicinal plants that help to retard post-prandial blood glucose
35 increase (Dalar and Konczak, 2013; Nair et al., 2013). They might be an
36 important supporting approach in the management of postprandial
37 hyperglycaemia. Pancreatic lipase is the key enzyme which hydrolyses
38 triglyceride into glycerol and fatty acids, facilitating an uptake of fat; thus, lipase
39 inhibitors hinder fat digestion and absorption (Grove et al., 2012; Zheng et al.,
40 2010).

41 Although phenolic compounds are considered major contributors to food
42 antioxidant capacity, other antiradical compounds with significant antioxidant
43 properties, such as melatonin (N-acetyl-5-methoxytryptamine), have been
44 described (Aguilera et al., 2016; Milczarek et al., 2010; Reiter et al., 2003). This
45 indolamine has been also quantified in some medicinal plants as feverfew (Murch
46 et al., 1997), St. John's wort (Murch et al., 2000), fennel, anise (Manchester et al.,
47 2000), Chinese medicinal plants (Chen et al., 2003), and Thai herbal teas
48 (Padumanonda et al., 2014), showing high melatonin levels. In addition, this
49 compound exhibits beneficial actions such a regulatory role on the energy
50 balance and antiobesity effect through the activation of brown adipose tissue
51 (Bonnefont-Rousselot, 2014; Cipolla-Neto et al., 2014). For these reasons, the
52 consumption of herbal infusions may be a successful strategy for protecting
53 against oxidative damage and closely related chronic diseases.
54 Little is still known about the bioactive compounds and antioxidant capacity in
55 herbal infusions. The aim of this work was to provide new knowledge on the
56 profile of the bioactive compounds and health promoting properties of nineteen
57 highly consumed herbal infusions. To achieve the goal, the content of melatonin
58 and phenolic compounds, antioxidant capacity, and *in vitro* inhibitory effect
59 against energetic metabolic enzymes named lipase, α-glucosidase, and α-amylase
60 were determined.

61 **2. Materials and Methods**

62 2.1. Botanicals

63 Nineteen commercial medicinal plants provided by a local company were
64 selected and classified into three groups according to the system that point out
65 their main function: nervous (relaxing and stimulating herbs), digestive, and

66 cardiovascular systems (Table 1). The flow diagram of the experimental design is
67 illustrated in Figure 1.
68 2.2. Preparation of herbal infusions
69 Infusions were prepared from the selected plants according to the method
70 described by Marques and Farah (2009), with some modifications. Briefly, 50 mL
71 of boiling water was added to 1g of plant material, extracted for 5 min and
72 filtered using a paper filter (11µm, Whatman).
73 2.3. Experimental procedures
74 *2.3.1. Melatonin*
75 It was assessed using a modified method based on the descriptions of Aguilera et
76 al. (2015). The evaporated methanolic extracts were dissolved in 2 mL of distilled
77 water, and ultrasonicated for 2 min. Samples were purified using solid phase
78 extraction (SPE, cartridge C-18, Waters). The obtained extracts were evaporated
79 to dryness by using an evaporator centrifuge (Speed Vac SC 200, Savant, USA).
80 The residues were dissolved in mobile phase. Melatonin was determined by
81 HPLC-ESI-MS/MS triple quadrupole (Varian 1200L with API-ES between 10
82 and 1500 Da range mass). Samples and standard solutions were analysed by
83 triplicate. Melatonin content was expressed as ng/g dry weight (DW).
84 *2.3.2. Total Phenolic Compounds (TPC)*
85 They were determined by Folin-Ciocalteu colorimetric method according to
86 Singleton et al. (1998). The results were expressed as gallic acid equivalents (mg
87 of GAE/g DW) and analysis were carried out in triplicate for each sample. In a
88 tube, 3 mL Milli-Q water, 50 µL sample and 250 µL Folin-Ciocalteau reactive
89 was merged. After 3 min of repose, 750 µL of Na_2CO_3 (20%) and 950 µL of

90 Milli-Q water were added, mixed and brought to darkness during 120 min.
91 Absorbance was measured at 760 nm.

92 *2.3.3. Total Flavonoids Content (TFC)*

93 The content was quantified according to Xu and Chang (2007). Briefly, 1mL
94 herbal infusion was mixed with 300µL of 5% $NaNO_2$ and samples were
95 incubated at room temperature for 5 min. Subsequently, 300µL of 10% $AlCl_3$ was
96 added and incubation continued for 6 min. Then, 2 mL of NaOH 1M was added,
97 vortexed and the absorbance was read at 510 nm. Total flavonoid content was
98 calculated with a calibration curve of catechin and the results were expressed as
99 mg CAE/g DW.

100 *2.3.4. DPPH assay: free radical scavenging activity*

101 DPPH free radical scavenging capacities of herbal infusions were evaluated
102 according to Xu and Chang (2007). Fifty µL of samples extracts were added to
103 1950 µL methanol solution of DPPH radical ($6 \cdot 10^{-5}$ mol/L). The mixture was
104 shaken vigorously and the decrease in absorbance (517 nm) was monitoring until
105 to obtain a steady state. The reaction was measured using methanol as blank. The
106 percentage of DPPH• inhibition in the steady state was determined as:

$$\% \text{ DPPH inhibition} = \frac{\text{Ac-At}}{\text{Ac}} \times 100$$

107 Where Ac is the absorbance of the control at time = 0 min; and At is the
108 absorbance of the sample at steady state. Trolox was used as a reference standard,
109 and the results were expressed by µmol TE/g dry weight.

110 *2.3.5. ABTS assay: radical scavenging assay.*

111 This antioxidant capacity assay was determined according to Re et al. (1999),
112 with slight modifications. Briefly, ABTS was dissolved in water to a 7mM
113 concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS

114 stock solution with 2.45 mM potassium persulfate (final concentration) and
115 allowing the mixture to stand in the darkness at room temperature for 12-16 h
116 before use. The $ABTS^{\bullet+}$ working solution was prepared by dilution with PBS
117 0.01 M pH=7.4 to an absorbance of 0.700 ±0.005 at 734 nm. All samples were
118 diluted to provide 20-80% inhibition of the blank. Twenty microliters of diluted
119 sample were mixed with $ABTS^{\bullet+}$ working solution (2000 µL) and after 30 min of
120 incubation at room temperature the absorbance of the reaction was measured at
121 734 nm (A sample). The absorbance of control (2000 µL $ABTS^{\bullet+}$ working
122 solution with 20 µL PBS) was recorded in advance (A control). The percent of
123 inhibition was calculated using the formula:

$$\% \text{ ABTS inhibition} = \frac{\text{Ac-At}}{\text{Ac}} \times 100$$

124 Where Ac is the absorbance of the control at time = 0 min; and At is the
125 absorbance of the sample at steady state. Trolox was used as a reference standard,
126 and the results were expressed by µmol TE/g dry weight.

127 *2.3.6. FRAP assay: ferric reducing antioxidant power assay*

128 Total reducing antioxidant capacity was performed using the FRAP assay as
129 previously described (Aguilera et al., 2016). 1.5 mL of a working FRAP reagent
130 [acetate buffer 0.3 M pH 3.6, 10 mM tripyridyl s-triazine (TPTZ) in 40 mM HCl
131 and 20 mM $FeCl3 \cdot 6H2O$ (10:1:1) (v/v/v)] were warmed to 37 °C, and then 50 µL
132 of samples were added. The absorbance was taken at 593 nm against reagent
133 blank after 10 min. FRAP values were calculated and expressed as µmol of
134 Trolox equivalents/ g DW (µmol TE/ g DW).

135 *2.3.7. ORAC Assay: oxygen radical absorbing capacity*

136 Oxygen radical scavenging capacity was determined as described previously
137 (Aguilera et al., 2014). Briefly, the reaction was carried out at 37 °C in 75 mM

138 phosphate buffer (pH 7.4) and the final assay mixture (200 µL) contained
139 fluorescein (70 nM), 2,2-azobis (2-methyl-propionamidine)-dihydrochloride (12
140 mM), and antioxidant standard (Trolox or sample extracts). Fluorescence was
141 read at 485 nm excitation and 520 nm emission. Fluorescence measurements
142 were normalized to the oxidation control (phosphate buffer) and stability control
143 (no antioxidant). Final ORAC values were expressed as µmol Trolox equivalents/
144 g DW (µmol TE/ g DW).

145 *2.3.8. Deoxyribose assay: hydroxyl radical scavenging assay*

146 This assay was performed on the basis of the method described by Halliwell et al.
147 (1987). In brief, 575 µL of sample, 25 µL of $FeCl_3$ (1 mM), 100 µL of EDTA
148 (1.04 mM), 100 µL of 2-Deoxy-ribose (28 mM), and 100 µL of H_2O_2 (28 mM)
149 were added. The reaction was initiated by mixing 100 µL of ascorbic acid (1
150 mM); total volume of the reaction was 1 mL. After incubation, for 1 hour at 37
151 ºC, the reaction was finished by 1 mL of TCA (1% (w/v)). The colour was then
152 developed by 1 mL TBA (2% (w/v) in NaOH (0.05M) and heating in and oven at
153 100ºC, for 15 min to develop colour due to MDA like a product of deoxyribose
154 damage. The reaction was cooled and the absorbance was read at 532 nm.
155 Mannitol was used as a standard; the results also were expressed as mmol eq
156 mannitol/g DW.

157 *2.3.9. Lipase inhibitory effect*

158 The inhibitory effect of the lipase was determined using and enzymatic kit
159 following the manufacturer's instructions (Lipase-PS™, Procedure No. 805,
160 Trinity Biotech, Jamestown, NY) adapted to micromethod. Substrate solution
161 (225 µL), lipase (3.75 µL) and samples (3.75 µL) were firstly incubated for 5 min
162 at 37 ºC. The activator reagent (75 µL) was subsequently added. Absorbance at

550 nm was measured after 2 min (initial) and 3 min (final) of incubation at 37 ºC. The percentage of α-glucosidase inhibition was calculated using the equation:

$$\text{Lipase inhibition (\%)} = \frac{A_{n.c} - A_s}{A_s} \times 100$$

Here $A_{n.c}$ is the A_{550nm} of the negative control and As is the A_{550nm} of the sample. All measurements were performed in triplicate.

2.3.10. α-Glucosidase inhibitory effect

α-Glucosidase enzyme was extracted previously to the assay. Briefly, 100 mg of rat intestine powder were dissolved in 3 mL of NaCl (0.9%), sonicated in an ice bath for 6 min and then centrifuged at 10000 g for 30 min. The supernatant containing the enzyme was stored in the freezer. In a 96-well microplate, 100 µL of sample dissolved in PBS 100 mM (pH 6.9) were mixed with 100µl of α-glucosidase (diluted 1/10) and 100 µL of 4-MUG (2mM). Fluorescence was then monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm for 30 min at 37 °C. Blank of sample and negative control (buffer, enzyme and 4-MUG) were included. Acarbose was used as a positive control. The percentage of α-glucosidase inhibition was calculated using the equation:

$$\text{α-glucosidase inhibition (\%)} = \frac{F_{nc} - F_s}{F_{nc}} \times 100$$

Where Fn.c. is the fluorescence of the negative control and Fs/cal of the sample. All measurements were performed in triplicate.

2.3.11. α-Amylase inhibitory effect

The α-amylase inhibitor content was determined by starch/iodine procedure of Piergiovanni (1992). Suitable aliquots of the extracts were mixed with α amylase solution and incubated at 40 °C for 30 min to allow formation of the inhibitor enzyme complex and the amount of remaining α amylase was then determined.

185 The absorbance was read at 565 nm. The results were expressed as μg inhibitor
186 content/100g DW.
187 2.4. Statistical Analysis
188 Each sample was analysed in triplicate. Data were expressed as mean ± standard
189 deviation (SD). The data were analysed by one-way analysis of variance
190 (ANOVA) and post-hoc Duncan's test. Differences were considered to be
191 significant at $p < 0.05$. Relationships between the analysed parameters were
192 evaluated by computing Pearson linear correlation coefficients setting the level of
193 significance at $p < 0.05$ and $p < 0.001$. Cluster analysis was used to construct
194 hierarchical dendrograms, searching the natural groupings among the samples.
195 The sample similarities were calculated on the basis of the squared Euclidean
196 distance. The statistical analysis was performed by SPSS 20.0.

197 **3. Results and Discussion**
198 3.1. Content of bioactive compounds in infusions
199 Melatonin was detected in all studied herbal infusions ranging from 5.6 to 232.3
200 ng/ g DW (Table 2). Chamomile infusion showed the highest melatonin content
201 while the lowest value was found in St. John's wort infusion. High values were
202 also found for green tea, valerian, and hawthorn (256-225 ng/g DW). Data were
203 similar to those reported by Chen et al. (2003), but different to those found by
204 other authors (Ansari et al., 2010; Padumanonda et al., 2014). These variations
205 could be due to the different plant materials and the subsequent melatonin
206 extraction, identification, and measurement (Cao et al., 2006; Garcia-Parrilla et
207 al., 2009). Several factors might influence the concentration of melatonin such as
208 stage of maturity, rate, and quality of light, the presence of phytochemicals, and
209 circadian characteristics of plants (Manchester et al., 2000; Paredes et al., 2009).

Melatonin contents in common herbal infusions might be considered as a dietary strategy to increase its plasmatic level, especially the digestive herbal infusion of chamomile, commonly used for its calming effect.

Total phenolic compounds ranged from 6.0 to 133.3 mg GAE/g DW, being lemon balm, the herbal infusion with the highest level and, chamomile, the lowest (Table 2). Values found for St. John's wort (72.9 mg GAE/g DW), boldo (62.1 mg GAE/g DW) and green tea (61.0 mg GAE/g DW) were similar to those previously reported in the literature (Albayrak et al., 2012). Green tea exhibited 2-fold high phenolic levels than red tea and 3-fold higher than black tea. These results may be explained based on the degradation of phenolics during tea oxidation process (Pereira et al., 2014). The high content found in boldo might be due to the gentle process used to dry the leaves (Soto et al., 2014). In general, it was observed that those herbal infusions from leaves and flowers (St. John's wort, green tea, and boldo) exhibited higher contents than those from roots (valerian). With regard to flavonoids, the contents ranged from 2.0 to 127.6 mg CAE/g DW, being the highest lemon balm which also showed the highest phenolic compounds; data were according to literature (Sakulnarmrat and Konczak, 2012). In most herbal infusions, flavonoid contribution was more than 60% to total phenols, except in case of green tea and horsetail (5 and 7%, respectively). Several factors might affect the phenolic content such as technological processes (plant processing, concentration, time, and temperature of extraction) (Horžić et al., 2009; Ramalho et al., 2013), type of plant (species, part used, stage of development), and environmental facts (climate, season, stresses among others) (Pereira et al., 2014).

234 According with the data hereby reported infusions commonly used for nervous
235 system valerian and green tea are good sources of melatonin and lemon balm is of
236 phenolic compounds and flavonoids; regarding to those used to treat the digestive
237 system chamomile contains high level of melatonin and boldo of phenols. In
238 addition, with respect to infusions dedicated to treat the cardiovascular system
239 hawthorn can be considered a good source of melatonin and rosemary of phenols
240 (Table 2).

241 3.2. Antioxidant capacity

242 Values obtained by DPPH, ABTS, FRAP, ORAC, and deoxyribose scavenging
243 assays, are shown in Figure 2. DPPH and ABTS assays were used to determinate
244 free-radical scavenging properties of herbal infusions. The DPPH values showed
245 lemon balm as outstanding free radical scavenging capacity (741.0 μmol TE/g
246 DW) while horsetail was the lowest (16.3 μmol TE/g DW) (Figure 2a).

247 High antioxidant capacity by DPPH method was found in infusions of St.
248 John's wort, green tea, boldo, and rosemary (463.5-363.6 μmol TE/g DW) made
249 using flowers and leaves. Results were higher than those found in the literature
250 (Wong et al., 2006). Total antioxidant capacity measured by the ABTS (Figure
251 2b) of green tea was the highest (1330.6 μmol TE/g DW) while that found for
252 horsetail was the lowest (81.4 μmol TE/g DW). High antioxidant capacity values
253 were found in lemon balm, boldo, and St. John's wort infusions (1170.4-886.8
254 μmol TE/g DW, respectively) which were in line with those reported by Cai et al.
255 (2004), Silva et al. (2007), and Li et al. (2013). Similar trends were obtained by
256 application of both antioxidant methods being those infusions used for treating
257 the nervous system such as lemon balm, green tea, and St. John's wort the best
258 natural sources of antioxidants and boldo for the digestive system the wort.

259 Data obtained by FRAP method are in line with those obtained by DPPH and
260 ABTS assays (Figure 2c). Lemon balm displayed the highest antioxidant capacity
261 (671.0 μmol TE/g DW) followed by green tea, St. John's wort, rosemary, boldo,
262 thyme (482.3-235.8 μmol TE/g DW), and horsetail (18.3 μmol TE/g DW).
263 ORAC values were consistently higher than those obtained for DPPH, ABTS,
264 and FRAP. Lemon balm exhibited the highest ORAC activity (1800.6 μmol TE/g
265 DW) (Figure 2d), it was found that 3 of the 19 herbal infusions contained more
266 than 850 μmol TE/g DW and 9 of them were below 60 μmol TE/g DW. In
267 particular, black tea exhibited the lowest oxygen radical absorbance capacity
268 (29.7μmol TE/g DW). Results were similar to those obtained by Silva et al.
269 (2007) and Soto et al. (2014). Oxygen free radicals are the most common free
270 radicals generated in the organism and, therefore, are accepted to be closer to
271 human physiology than any other reagent-based antioxidant testing method
272 (Badarinath et al., 2010).
273 Deoxyribose assay was used to determine the hydroxyl radical scavenging
274 activity. All infusions showed medium-high inhibitory capacity against
275 deoxyribose damage (DR) (Figure 2e), interestingly through this methodology,
276 fennel exhibited a relevant level (59.1 nmol mannitol/ g DW). In addition, the
277 highest DR degradation inhibitory activity (96.0%) was again showed by lemon
278 balm. Higher inhibitory values were obtained than those reported in the literature
279 (Gião et al., 2008). There was a dose-dependent relationship between the
280 concentration of infusion and inhibition (Gonçalves et al., 2013).
281 Lemon balm, green tea, and boldo infusions showed remarkable antioxidant
282 capacity by the different methods. As expected, data are in agreement with those
283 above reported regarding to the composition in antioxidant phytochemicals.

284 Flavonoids, such as catechins may be the main contributors to the antioxidant
285 potency of these infusions (Quesille-Villalobos et al., 2013; Quezada et al.,
286 2004).

287 3.3. Inhibitory effect against digestive enzymes

288 Identification of the sources of inhibitors of digestive enzymes has been
289 undertaken. Literature reported rich-phenolic compound foodstuffs are the source
290 of inhibitors of pancreatic lipase (Garza et al., 2011). Obesity was found to be
291 partially associated with low antioxidant status (Li et al., 2013) and thus, phenolic
292 compounds possessing high antioxidant capacity may useful for preventing
293 obesity. The inhibitory effect of the infusions against lipase activity ranged from
294 73% for boldo to 18% for passionflower (Table 3). In addition, green tea showed
295 also pronounced inhibitory effect against the enzymatic activity of pancreatic
296 lipase (71%). Most of the infusions (n=14) displayed moderate inhibitory effect
297 of lipase activity (20-60%). Horsetail, valerian, and passion flower inhibited
298 lipase activity in less than a 20%. Results indicated that all phenolic-rich
299 infusions are effective inhibitors of lipase activity in agreement with literature
300 (Sharma et al., 2005; Zheng et al., 2010). Phenolic compounds present in the
301 infusions might be responsible for the suppression of pancreatic lipase although
302 this effect may be affected by the presence of other compounds, beside phenolics,
303 which possess enzyme inhibitory activity (Dalar and Konczak, 2013; Gironés-
304 Vilaplana et al., 2013). Digestive and stimulating infusions, boldo and green tea,
305 have potential for treating or reducing risk of obesity caused by a high-fat diet.

306 Boldo, St. John's wort, lemon balm, green tea, and senna exhibited potential
307 antidiabetic effect since they inhibited α-glucosidase activity in about 98% (Table
308 3). The other 12 infusions also strongly decreased lipase activity (> 70%). In

309 general, all infusions were effective inhibitors of α-glucosidase activity, being
310 higher than those found in other widespread herbal infusions in other world
311 regions. α-Glucosidase is an enzyme that hydrolyses starch and disaccharides to
312 glucose, therefore, α-glucosidase inhibitors could retard hydrolysis of dietary
313 carbohydrates, reduce the bioavailability of glycaemic sugar, and improve sugar
314 tolerance. Our results seem to indicate that the digestive herbal infusion called as
315 boldo and relaxing infusion named as lemon balm could be good natural
316 beverages to treat diabetes due to its α-glucosidase inhibitory effect.

317 α-Amylase acts upon large polysaccharides and breaks them into single units
318 of sugars. Natural α-amylase inhibitors offer an attractive therapeutic approach in
319 the treatment of postprandial hyperglycaemia. Regarding the content of α-
320 amylase inhibitors in the infusions, it was not observed in most of the samples.
321 However, St. John's Wort, black tea, and boldo presented enzyme inhibitors 5.51,
322 0.81, and 4.07 µg inhibitor/ 100 g DW, respectively. Similar inhibitory effects
323 were reported for other herbal infusions (Ranilla et al., 2010; Sakulnarmrat and
324 Konczak, 2012; Subramanian et al., 2008). The results suggest that boldo and St.
325 John's Wort are composed of more effective inhibitors of the enzyme than the
326 rest of infusions. This effect may be mainly ascribed to phenol profiles. Several
327 studies suggest that plant polyphenols act as inhibitors of carbohydrate
328 hydrolysing enzymes (Boath et al., 2012). Therefore, these infusions have
329 potential use in health as an antidiabetic agent.

330 3.4. Correlation between content of bioactive compounds and infusions´ properties

331 Correlation of different parameters analysed in the present study is presented in
332 Table 4. As expected, positive significant correlation (r= 0.890; $p < 0.01$) was
333 detected between total phenolics and flavonoids. The phenolic contents correlated

334 with the antioxidant capacity of the infusions measured by the different methods
335 (DPPH r= 0.963; ABTS r= 0.862; FRAP r= 0.954; ORAC r= 0.850; $p < 0.01$). A
336 highly significant positive correlation was also observed between values of total
337 flavonoids and antioxidant capacity, highlighting DPPH r= 0.854 and FRAP r=
338 0.819 ($p < 0.01$). Then, phenolic compounds scavenged oxygen free radical
339 contributing to the overall antioxidant capacity of the infusions.

340 A significant positive correlation between content in total phenolic compounds
341 and inhibitory effect against lipase activity was observed. Moreover, a similar
342 trend was observed for TFC and inhibitory effect of lipase activity. These results
343 are in agreement with Ranilla et al. (2010) and Dalar et al. (2013) who found
344 similar correlations between lipase inhibitory activity and phenolic content of
345 infusions commonly used in Latin America and Eastern Anatolia.

346 A positive significant correlation between total phenolics and inhibition of α-
347 glucosidase was observed. The content of flavonoids also correlated with α-
348 glucosidase inhibitory effects. Different phenolic compounds found in herbal
349 infusions may be responsible of this antidiabetic effect. In this sense, studies have
350 showed that chlorogenic acid and anthocyanins, present in the infusions, possess
351 a high α-glucosidase inhibitory effect (Boath et al., 2012).

352 No significant correlation between data on TPC, TFC and inhibitory effect of
353 amylase was found. Data are in line with those previously reported in the
354 literature (Dalar and Konczak, 2013; Ranilla et al., 2010). The formation of
355 protein-phenolic compound complexes, which is the prerequisite of enzyme
356 inhibition, depends on the molecular structure of phytochemicals and the
357 conformation of proteins (Dalar and Konczak, 2013).

From the dendrogram (Figure 3), two well-defined clusters can be identified as follows: the first cluster (A) is composed of three samples (16% of herbal infusions), and the second cluster (B) contains the remaining samples (84% of herbal infusions). The cluster A is formed by those infusions showing high levels of bioactive compounds, antioxidant capacity, and inhibitory effect against lipase and glucosidase (green tea, boldo, and lemon balm). The cluster B corresponds to rest of samples that presented medium and low values in studied assays. Interestingly St. John's Wort was very close to cluster A. These observations are in line with those shown in Table 4.

4. Conclusions

In summary, for the first time our findings support the potential health properties of infusions for nervous (green tea and lemon balm) and digestive (boldo) systems as alternative medicine for preventing and treating chronic diseases. These infusions are rich in melatonin, phenolic compounds, isoflavones, and inhibitors of lipase and α-glucosidase. Correlation studies seem to indicate phytochemicals contribute to the antioxidant, antidiabetic, and antiobesity potential effects of these infusions. The health benefits derived from the intake of these infusions are, until now, uncertain as it is not recognized if their chronic consumption through the diet have physiological effects, thus further studies are required.

Abbreviations

AUC	Area Under Curve
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
CAE	Catechin Equivalents

382	DPPH	2,2-diphenyl-1-picrylhydrazyl
383	DW	Dry Weight
384	FRAP	Ferric Ion Reducing Antioxidant Power
385	GAE	Gallic Acid Equivalents
386	MRM	Multiple Reaction Monitoring
387	ORAC	Oxygen Radical Absorbance Capacity
388	PBS	Phosphate Buffer Solution
389	SPE	Solid Phase Extraction
390	TE	Trolox Equivalents
391	TFC	Total Flavonoids Content
392	TPC	Total Phenolic Compounds

393 **Conflict of interest**

394 The authors declare no conflict of interest.

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548 **Table 1.** Botanical names, families, medicinally used parts, and traditional uses of 19 selected medicinal plants.

	Herbal Infusions	Scientific name	Family	Tissues used for herbal infusion	Traditional use
Nervous System	Lemon balm	*Melissa officinalis L.*	*Lamiaceae*	Stems, leaves, and flowers	Relaxing
	Linden	*Tilia platyphyllos scop*	Malvaceae	Inflorescences	Relaxing
	Passionflower	*Passiflora incarnata* L.	*Passifloraceae*	Leaves and flowers	Relaxing
	St. John´s Wort	*Hypericum perforatum* L.	*Clusiaceae*	Flowers	Relaxing
	Valerian	*Valeriana officinalis* L.	*Caprofoliaceae*	Roots, rhizomes, and stolons	Relaxing
	Black Tea	*Camellia sinensis* L.	*Theaceae*	Leaves	Stimulant
	Green Tea	*Camellia sinensis* L.	*Theaceae*	Leaves	Stimulant
	Red Tea	*Camellia sinensis* L.	*Theaceae*	Leaves	Stimulant
Digestive System	Boldo	*Peumus boldus molina*	*Monimiaceae*	Leaves and cortex	Choleretic and cholagogue
	Chamomile	*Matricaria chamomilla* L.	*Asteraceae*	Flower heads	Antispasmodic
	Fennel	*Foeniculum vulgare miller*	*Apiaceae*	Fruits, seeds, root, and leaves	Antiflatulent or carminative
	Green Anise	*Pimpinella Anisum* L.	*Apiaceae*	Fruits	Antiflatulent or carminative
	Pennyroyal	*Mentha pulegium* L.	*Lamiaceae*	Leaves	Carminative
	Senna	*Cassia Angustifolia Vahl*	*Fabaceae*	Leaves and fruits	Laxative
Cardiovascular System	Hawthorn	*Crataegus oxyacanthal*	*Rosaceae*	Floral tops, leaves, fruits, and cortex	Antiarrhythmic and hypotensive
	Horsetail	*Equisetum arvense* L.	*Equisetaceae*	Stem	Antihemorrhagic and hematinic
	Olive Tree	*Olea europaea* L.	*Oleaceae*	Leaves and fruits	Antiarrhythmic and hypotensive
	Rosemary	*Rosmarinus officinalis* L.	*Lamiaceae*	Leaves, stems, and flowers	Hypertensive
	Thyme	*Thymus vulgaris* L.	*Lamiaceae*	Leaves and flowers	Hypertensive

549 **Table 2.** Melatonin (ng/g DW), total phenolic content (mg GAE/g DW), and total
550 flavonoid content (mg CAE /g DW) of herbal infusions.

Herbal infusions	Melatonin (ng/g DW)	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)
Nervous System			
Relaxing			
Lemon Balm	99.9 ± 3.1 ^{e}	133.3 ± 6.9 ^{k}	127.6± 2.7 ^{l}
Linden	77.6 ± 0.8 ^{c}	33.9 ± 3.2 ^{f}	11.8 ± 0.1 ^{e}
Passion Flower	95.1 ± 5.2 ^{e}	14.9 ± 1.2 ^{b}	2.0 ± 0.3 ^{a}
St. John´s Wort	5.6 ± 0.1 ^{a}	72.9 ± 2.5 ^{j}	45.7 ± 2.4 ^{i}
Valerian	232.3 ± 6.8 ^{l}	9.8 ± 0.3 ^{a}	2.8 ± 0.5 ^{a}
Stimulating			
Black Tea	5.6 ± 0.3 ^{a}	21.5 ± 0.4 ^{d}	5.1 ± 0.0 ^{bc}
Green Tea	256.4 ± 6.3 ^{m}	61.0 ± 1.3 ^{i}	2.9 ± 0.2 ^{a}
Red Tea	106.6 ± 2.9 ^{f}	30.0 ± 1.6 ^{e}	14.5 ± 0.6 ^{f}
Digestives			
Boldo	120.1 ± 3.5 ^{g}	62.1 ± 0.2 ^{j}	51.0 ± 3.2 ^{j}
Chamomile	298.6 ± 7.1 ^{n}	6.0 ± 0.4 ^{a}	3,7 ± 0.1 ^{ab}
Fennel	ND	19.9 ± 0.8 ^{cd}	6.4 ± 0.6^{c}
Green Anise	115.6 ± 3.5 ^{g}	15.9 ± 0.4^{b}	9.0 ± 0.2 ^{d}
Pennyroyal	136.1 ± 4.0 ^{h}	16.2 ± 0.7 ^{bc}	14.0 ± 0.7 ^{f}
Senna	86.2 ± 2.3 ^{d}	6.8 ± 0.1 ^{a}	3.5 ± 0.1 ^{ab}
Cardiovascular System			
Hawthorn	224.6 ± 5.6 ^{k}	29.2 ± 1.0 ^{e}	17.8 ± 0.5 ^{g}
Horsetail	197.8 ± 4.7 ^{j}	29.9 ± 1.1^{e}	2.0 ± 0.1 ^{a}
Olive Tree	101.4 ± 2.6 ^{ef}	26.7 ± 0.9 ^{e}	18.4 ± 2.2 ^{g}
Rosemary	61.2 ± 1.6 ^{b}	47.4 ± 1.3 ^{h}	51.5 ± 0.9 ^{k}
Thyme	154.3 ± 4.4 ^{i}	40.3 ± 0.7 ^{g}	35.9 ± 1.6 ^{h}

551 Results are reported as mean ± SD (n=3). N.D. No detected. Mean values of each herbal
552 infusion followed by different superscript letters significantly differ when subjected to
553 Duncan´s multiple range test ($p < 0.05$)

554 **Table 3.** Inhibitory effect against lipase, α-glucosidase, and α-amylase activities.

Herbal infusion	Lipase (% Inhibition)	α-glucosidase (% inhibition)	α-amylase (µg inhibitor/100 g DW)
Nervous System			
Relaxing			
Lemon Balm	63.4 ± 1.7 ^{hi}	98.1 ± 0.5 ^{fgh}	ND
Linden	61.7 ± 5.9 ^{gh}	95.0 ± 1.0 ^{efgh}	0.30 ± 0.01 ^{b}
Passion Flower	-17.7± 0.8 ^{a}	94.8 ± 2.3 ^{efgh}	ND
St. John´s Wort	57.7 ± 4.9 ^{fgh}	98.2 ± 0.2 ^{h}	5.51 ± 0.29 ^{e}
Valerian	11.4 ± 1.2 ^{b}	74.3 ± 5.7 ^{b}	ND
Stimulating			
Black Tea	45.4 ± 3.6 ^{ef}	93.9 ± 0.9 ^{efg}	0.81 ± 0.01 ^{c}
Green Tea	71.1 ± 1.0 ^{ij}	96.7 ± 0.2 ^{fgh}	0.26 ± 0.02 ^{ab}
Red Tea	62.3 ± 3.6 ^{ghi}	83.5 ± 1.6 ^{d}	ND
Digestives			
Boldo	72.8 ± 2.7 ^{j}	98.0 ± 1.8 ^{gh}	4.07 ± 0.16 ^{d}
Chamomile	34.7 ± 1.2 ^{c}	89.8 ± 7.8 ^{efgh}	ND
Fennel	34.3 ± 3.0 ^{cd}	86.8 ± 2.8 ^{d}	ND
Green Anise	46.3 ± 2.4 ^{de}	86.0 ± 1.5 ^{d}	ND
Pennyroyal	64.0 ± 2.0 ^{hij}	70.0 ± 3.7 ^{a}	ND
Senna	22.5 ± 0.0 ^{b}	96.7 ± 0.2 ^{fgh}	ND
Cardiovascular System			
Hawthorn	53.9 ± 5.0 ^{fg}	91.5 ± 0.9 ^{e}	0.37 ± 0.03 ^{b}
Horsetail	14.6 ± 1.2 ^{b}	79.7 ± 3.0 ^{c}	ND
Olive Tree	65.7 ± 2.0 ^{hij}	94.0 ± 1.6 ^{efg}	ND
Rosemary	58.3 ± 6.2 ^{fgh}	93.7 ± 0.6 ^{efg}	0.09 ± 0.02 ^{a}
Thyme	57.4 ± 1.0 ^{fgh}	92.8 ± 1.0 ^{ef}	ND

555 Results are reported as mean ± SD (n=3). N.D. No detected. Mean values of each herbal
556 infusion followed by different superscript letters significantly differ when subjected to
557 Duncan´s multiple range test ($p < 0.05$)

28

558 **Table 4.** Matrix of Pearson correlation coefficients between bioactive compounds found in herbal infusions, antioxidant capacity measured by
559 different methods, and enzymes inhibitory effect.

	TPC	TFC	DPPH$^{\bullet}$	ABTS$^{\bullet+}$	FRAP	ORAC	Deoxyribose	Lipase ‡	α-glucosidase ‡	α-amylase †
Melatonin	-0.232	-0.295[a]	-0.238	-0.146	-0.107	0.015	-0.318[a]	-0.193	-0.241	0.052
TPC		0.890[b]	0.963[b]	0.862[b]	0.954[b]	0.850[b]	0.594[b]	0.510[b]	0.451[b]	0.020
TFC			0.854[b]	0.610[b]	0.819[b]	0.754[b]	0.692[b]	0.432[b]	0.333[b]	0.047
DPPH$^{\bullet}$				0.893[b]	0.964[b]	0.760[b]	0.506[b]	0.604[b]	0.508[b]	-0.047
ABTS$^{\bullet+}$					0.912[b]	0.770[b]	0.282[a]	0.578[b]	0.581[b]	-0.032
FRAP						0.806[b]	0.512[b]	0.571[b]	0.477[b]	0.108
ORAC							0.518[b]	0.337[a]	0.366[b]	0.016
Deoxyribose								-0.021	0.166	0.433
Lipase									0.233	-0.466[a]
α-glucosidase										-0.035

560
561 ‡ Lipase and α-glucosidase inhibitory activities are expressed as % Inhibition

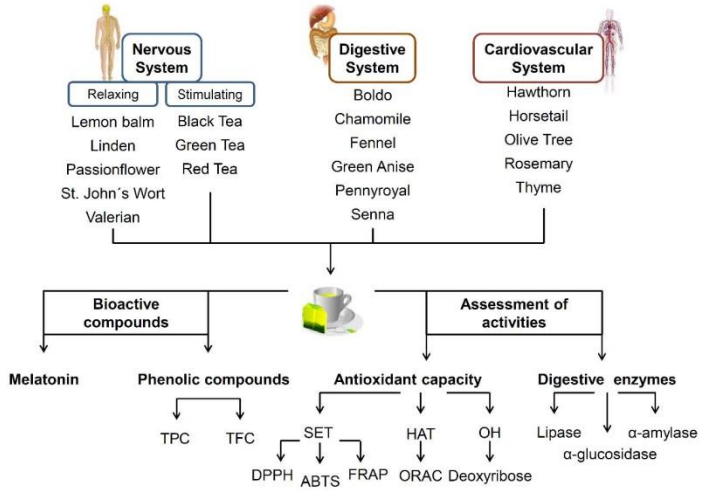
562 † α-Amylase inhibitory activity is expressed as μg inhibitor/100 g DW

563 [a] Significant at $p < 0.05$

564 [b] Significant at $p < 0.01$

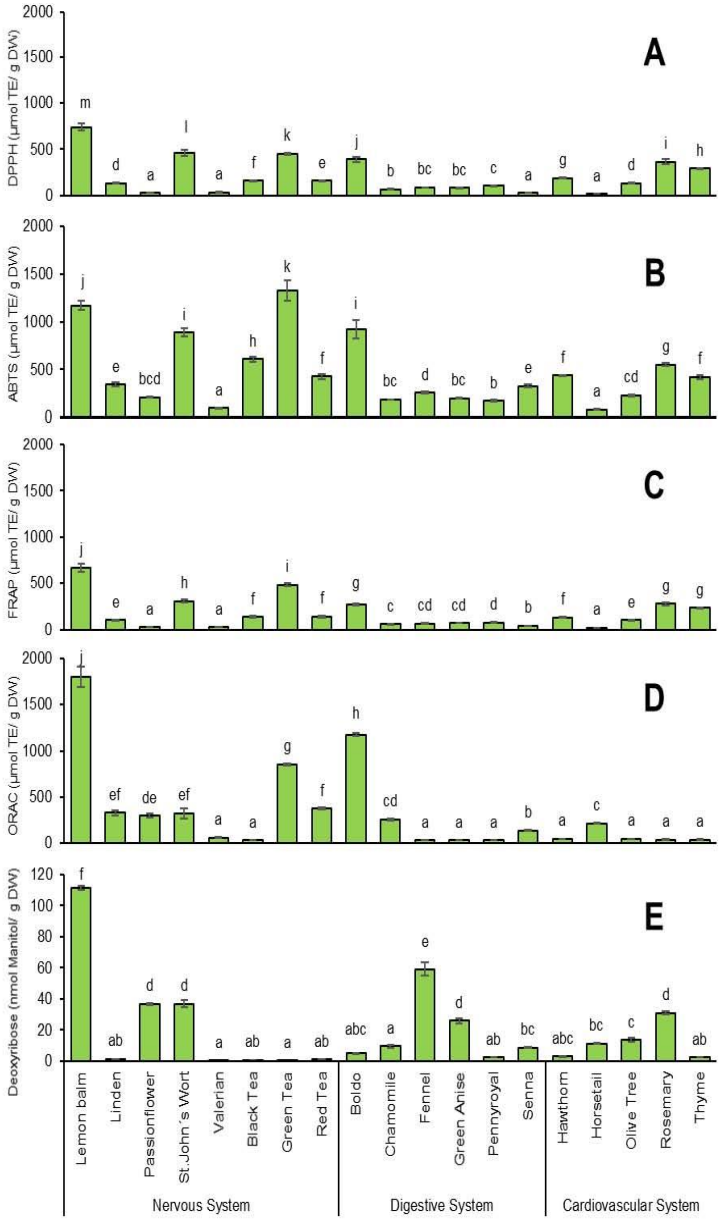
Figure

Figure 1



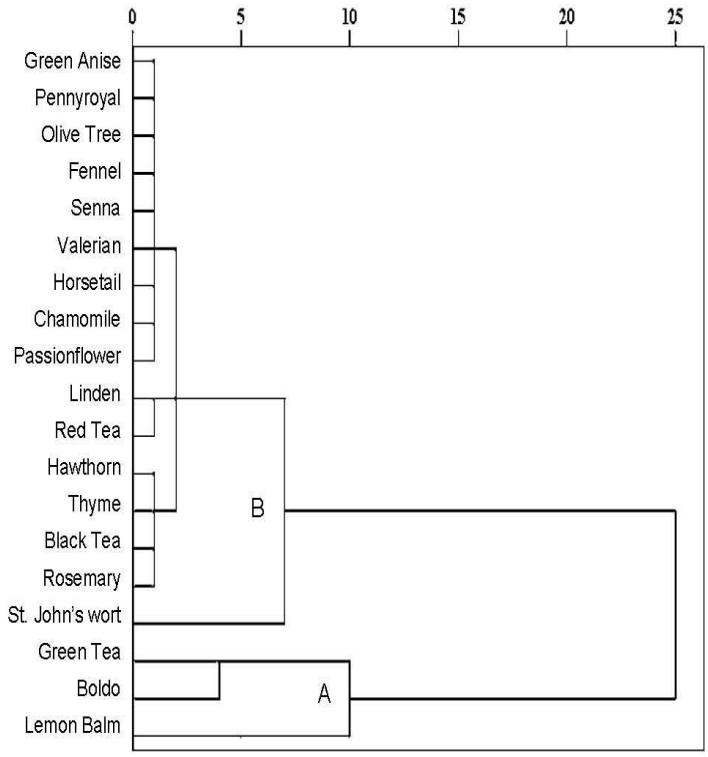
Figure

Figure 2



Figure

Figure 3



Figure

FIGURE CAPTIONS

Figure 1. Flow-diagram of the experimental design.

Figure 2. Antioxidant capacity of different herbal infusions measured by different methodologies: DPPH (a), ABTS (b), FRAP (c), ORAC (d), and Deoxyribose assay of herbal infusions. Bars with different letters significantly differ when subjected to Duncan's multiple range test ($p < 0.05$).

Figure 3. Dendrogram of hierarchical cluster analysis of herbal infusions. The variables included were melatonin, phenolic compounds and flavonoids concentrations, DPPH, ABTS, FRAP, ORAC and Deoxyribose assay values of antioxidant capacity, and lipase, α-glucosidase, and α-amylase inhibitory effects.